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**Standardization of  $I^{131}$  Solutions by Direct Comparison of Gamma Activities\*** (18079)

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Since no generally accepted procedure has been published for the standardization of  $I^{131}$  solutions for clinical use, it seems advisable to report a simple method in use in our laboratories. This procedure, which involves direct comparison of gamma-ray intensities of unknown and standard radioiodine solutions, is free from many uncertainties involved in the preparation and measurement of solid  $I^{131}$  samples, namely, (1) the danger of loss of activity by volatilization of iodine, (2) the uncertainties in geometry resulting from poor reproducibility in depositing the solid material, (3) the approximate nature of the corrections applied for absorption of beta particles in sample, air and counter window, and (4) the uncertainty of the primary geometry standardization (*e.g.* with RaD, E, F standard). These uncertainties are still present in the Bureau of Standards value for their iodine standard.

The standard  $I^{131}$  solution employed in the procedure described here can be obtained periodically from the Bureau of Standards.<sup>†</sup> They consist of approximately 3 ml of an aqueous solution, containing a known concentration of  $I^{131}$  usually in the order of 0.1 rutherford (rd) per ml. An accurately meas-

ured volume of this solution is transferred to a 5 ml glass ampoule and the volume made up to 3 ml and the ampoule sealed in a Bunsen flame. A 3.0 ml sample of the unknown solution containing between 0.1 and 3 rd per ml is prepared in the same manner using an identical ampoule.<sup>‡</sup> The gamma activities of the 2 samples are then compared by counting at the greatest practical distance from a standard Geiger-Mueller (G-M) counter, such that the counting rate of the weaker sample is greater than 100 counts per minute (c/m). To eliminate entirely the beta particles from  $I^{131}$ , the tube window should be covered with about 200 mg/cm<sup>2</sup> of aluminum.

Standard and unknown are counted in precisely identical positions with respect to the tube, each for a period which yields a total of greater than 10,000 counts whenever practical, so that the statistical error of the counts is negligible.

The arrangement of G-M tube and sample employed in this laboratory are shown in Fig. 1. The use of a rather large distance between sample and tube minimizes geometry fluctuations. The counting rate should not fall below about 100 c/m above background, however, in order that reliable counting data are obtained. Preparation of duplicate un-

\* Journal Series No. 915, University of Arkansas.

† U. S. Department of Commerce, Bureau of Standards, Radioactivity Section, Washington, D. C.

‡ Obtained from E. Macklett and Son, 220 East 23rd Street, New York City.

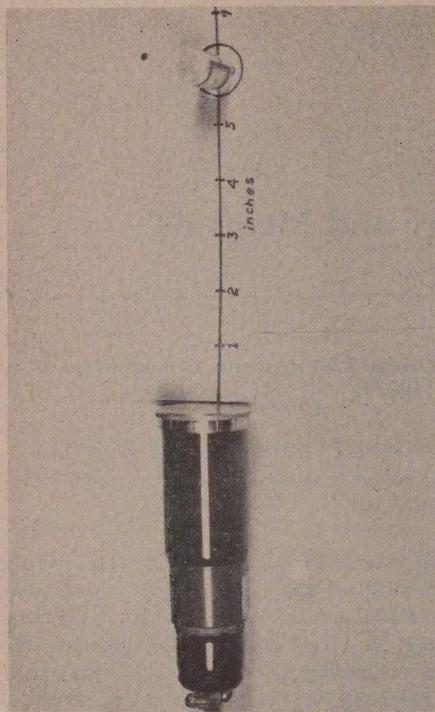
STANDARDIZATION OF I<sup>131</sup> SOLUTIONS

FIG. 1.

known samples, to minimize sampling errors, is advised.

Correction for decay of the 8 day I<sup>131</sup> in the standard solution (since standardization) must be applied in calculating the strength of the unknown. A sample measurement is described below:

A solution of about 2 millicuries (mc) of I<sup>131</sup> from Oak Ridge was diluted to 10 ml, yielding a concentration of about 7.4 rd/ml (1 mc = 37 rd). A sample of 1.0 ml of the unknown was placed in a 5 ml ampoule, 2.0 ml of water was added, and the ampoule sealed. A standard I<sup>131</sup> solution 9 days old was on hand; in the 9 day period, the activity of this standard had decayed from 0.1 to 0.046 rd/ml. The 2 samples were then compared as described above at a distance of 6 inches from the window of a Tracerlab TGC-2 tube, attached to a Nuclear Instrument Company, Model 165, scaling circuit. The counting rates so obtained are employed to calculate the activity of the unknown (X) from

the simple proportion:

$$X = \frac{(\text{activity of standard in rd}) (\text{c/m unknown})}{(\text{c/m standard})}$$

Application of the proper correction factor for dilution then yields a value for the actual activity of the unknown solution in rd/ml. Example: The standard had decayed for 9 days when used to standardize the unknown. As calculated above the standard then contained 0.046 rd/ml or 0.138 rd in 3 ml. This

0.138 rd

corresponds to 0.00373 mc ( $\frac{0.138 \text{ rd}}{37 \text{ rd/mc}}$ )

0.00373 mc) or 3.73  $\mu$ c. The standard gave 100 c/m at 6 inches with a background of 25 c/m. This minus background was 75 c/m. The unknown gave 3512 c/m at 6 inches. This minus background was 3487 c/m.

$$X = \frac{.00373 \text{ mc} \times 3487 \text{ c/m}}{75 \text{ c/m}} = 0.1734 \text{ mc}$$

This value represents the I<sup>131</sup> content of 1 ml of the original solution or 1/10 of the shipment from Oak Ridge. Thus the entire shipment contained 1.734 mc of I<sup>131</sup>.

The actual variability encountered in making repeated determinations on the same solution, *i.e.*, the statistical fluctuations of the method is presented in Table I. The tests were made more severe by comparing samples of different strengths (a 30-fold range of intensities) with a single I<sup>131</sup> standard. An evaluation of the accuracy of the method was attempted by comparing the values so obtained with an independent standardization of the solution against a RaD beta standard.

A 50 ml stock solution of radioiodine, containing approximately 10 rd of I<sup>131</sup>, was prepared. Samples of this solution were made up in duplicate according to the above procedure. The gamma activities of these samples were compared with a standard consisting of 2.00 ml of the calibrated I<sup>131</sup> solution provided by the National Bureau of Standards. At the time of its use the standard contained 0.0522 rd of I<sup>131</sup> as calculated from the data supplied with the standard solution. The low activity of the standard made it necessary to conduct the measurements at a distance of only 2 inches from

TABLE I. Statistical Variability in the Standardization of  $I^{131}$ .

ml of $I^{131}$ sol. taken*	Activity in c/m minus background	Activity in rutherford	Total activity of $I^{131}$ soln. in rd.†	Deviation from mean
.1	41 ± 2	.0187	9.35 ± .47	-.25
.1	40 ± 2	.0184	9.20 ± .46	-.40
.5	197 ± 5	.0906	9.06 ± .23	-.54
.5	207 ± 5	.0954	9.54 ± .23	-.06
1.0	402 ± 5	.185	9.25 ± .12	-.35
1.0	402 ± 5	.193	9.65 ± .11	+.05
2.0	863 ± 12	.398	9.95 ± .14	+.35
2.0	869 ± 12	.400	10.00 ± .14	+.40
3.0	1306 ± 15	.601	10.00 ± .12	+.40
3.0	1300 ± 15	.598	9.96 ± .11	+.36
Standard (2.0)	113 ± 3	.0522‡		
			Mean	9.60 ± .11§
Standardized against RaD beta standard†			7.93 ± .16	
			7.80 ± .20	
		Mean	7.87	

\* Make up to a total volume of 3.0 ml with distilled water.

† Stand. dev. based on counting error only.

‡ Calculated from data supplied by the National Bureau of Standards.

§ Stand. dev. of the mean for 10 samples.

the Geiger-Mueller tube. Even at this close distance geometry errors were less than 2% as shown by repeated counts on the same sample following repositioning of the ampoule.

It is seen from Table I that for low intensity samples the counting precision appeared to be the limiting source of error while for higher intensity samples other factors such as measurement of volume become more predominant. The agreement between the mean values obtained by the 2 methods of standardization was considered to be satisfactory, in view of the fact that small differences in the geometries and absorption coefficients of the  $I^{131}$  beta sample and RaD

standard were in the direction to give a low result.

*Summary.* A rapid and convenient method for the standardization of  $I^{131}$  solutions is described. A statistical evaluation of the method showed that the standard deviation of the mean for 10 samples was approximately 1%.

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## Maintenance of Pregnancy in Hypophysectomized Rats with Placental Implants.\* (18080)

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Using the placentoma reaction Astwood and Greep(1) demonstrated that the rat placenta

contains a luteotrophic substance capable of stimulating the corpora lutea to secrete pro-

\* Aided by grants from the Research Board of the University of California.

1. Astwood, E. B. and Greep, R. O., PROC. SOC. EXP. BIOL. AND MED., 1938, v38, 713.

gestin. No mention was made of the age of the placentae used, but it might be inferred from the work of Pencharz and Long(2) that the placenta of mid-gestation is able to take over the function of secreting gonadotrophins comparable to those of the pituitary. In the following experiment in which pregnancy was maintained in hypophysectomized rats during the critical 6-12 day phase further proof of a chorionic luteotrophin is presented; and support is also lent to Selye's indirect evidence for a substance in the rat placenta which stimulates the ovary to secrete estrin(3).

**Experimental.** Nulliparous, Long - Evans rats, 2-7 months old were used. Rats in proestrus were caged with a male in the afternoon and when sperm were identified in the vaginal smear on the following morning this was considered day 1 of pregnancy. The test rats or recipients were hypophysectomized<sup>†</sup> on day 6 and injected or implanted from day 6 to 11. Necropsy was performed on day 12. Oöphorectomy in a control experiment was performed immediately after hypophysectomy. Tests were made on placentae taken from donors on days 10, 12 and 15 of gestation. Average weights of such placentae were 38, 55 and 250 mg respectively. Sixty tests were made on different amounts of placental tissue from the 3 stages of pregnancy. In some instances the placental implants were supplemented by subcutaneous injections of estrone<sup>‡</sup> in sesame oil. In a few experiments, tests were made on separated fetal and decidual parts of the implantation mass. Except as noted in the Table the placentae were implanted within an hour of the removal from the donors. Two types of controls were used, namely, rats receiving no implants, and rats oöphorectomized as well as hypophysectomized receiving implants. For

2. Pencharz, R. and Long, J. A., *Am. J. Anat.*, 1933, v53, 117.

3. Selye, H., McKeown, T., *Proc. Roy. Soc. (B)*, 1935, v119, 1.

<sup>†</sup> We are greatly indebted to Mrs. Luree Hughes for hypophysectomizing these animals.

<sup>‡</sup> The estrone and progesterone used in this study were generously supplied through the courtesy of Dr. Daniel A. McGinty of Parke, Davis and Company.

TABLE I. Effect of Various Hormonal Combinations on Maintenance of Pregnancy in Rats Hypophysectomized on Day 6, Treated for 6 Days and Sacrificed on Day 12.

No. of rats	Placentae implanted daily	Day of gestation of donor	Dose of estrone	Rats with living emb.
6	0	—	—	0
6	2 whole	10	1 µg day 7	0
12	1-4 whole	12	—	3
10	5-8 "	12	—	8
3*	5-7 "	12	—	0
7	1 "	12	1 µg day 7, 9	5
3†	2 "	12	.5 µg daily	1
5†	2 fetal	12	.5 " "	2
5†	1-2 maternal	12	.5 " "	2
4	1-2 whole	15	—	0
4	1 "	15	1 µg day 7, 9	0
1	2 "	15	1 " " 9	1

\* Rats also oöphorectomized.

† Placentae used in these cases had been stored in a frozen condition 1-14 days.

confirmatory and comparative purposes, pituitary lactogenic hormone alone and with estrone was injected into another series of similar test animals. As shown earlier(4) lactogenic hormone did not maintain pregnancy unless combined with estrone.

**Results.** The results of the various experiments may be seen in Table I. Pregnancy was considered to have been maintained only when fetal viability was demonstrable at necropsy. When injections or implants were inadequate, blood and mucus always appeared in the vagina in more copious amounts than seen in the usual placental sign. In untreated controls, in implanted, oöphorectomized rats, and in rats implanted with placentae from the tenth day of gestation, this usually occurred on day 8 of pregnancy, within 2 days of hypophysectomy. In other rats inadequately treated, bleeding in excess of the placental sign was detected between days 9 and 12. At necropsy such rats showed evidence of partial success in maintaining the conceptus by the presence of placentae of various sizes and in some instances dead fetuses.

None of the uninjected rats, nor the injected rats that had been oöphorectomized

4. Lyons, W. R., Simpson, M. E. and Evans, H. M., *PROC. SOC. EXP. BIOL. AND MED.*, 1943, v52, 134.

as well as hypophysectomized maintained their embryos or placentae. During the progress of the experiment it became evident that the 12-day implantation sites, preponderantly decidual, were adequate in maintaining gestation if a sufficient number were implanted. Generally 5 or more of these placentae or approximately 275 mg daily were required. Double this weight of 15-day placentae proved ineffective. It was then decided to limit the experiment to the relatively potent 12-day placenta and to attempt to enhance its pregnancy-maintaining value by the addition of estrone. Histologic study of the ovaries, uteri and vaginae of the rats receiving only a single 12-day implant showed evidence of luteotrophic action even though pregnancy was not maintained. This was also true of the animals receiving purified lactogenic hormone alone in this and in the earlier series. When the rats were injected with 0.5-1  $\mu\text{g}$  of estrone daily as well as lactogenic hormone, pregnancy was maintained. As may be observed in Table I this dose was equally efficacious when given with a single 12-day placenta. In an attempt to localize the luteotrophic activity in the parts of the placenta composed mainly of either decidual or trophoblastic tissue, it was found possible to make a separation of the 12-day sites by discarding the embryo in its amnion and lifting the small trophoblastic part or *träger* away from the cupped, decidua basalis. The latter in turn was readily isolated by forceps as a compact button. Histologic sections of such parts of the placentae showed very few small nests of trophoblast in the decidual buttons and predominantly trophoblastic cells in the fetal part. However the latter also contained yolk sac epithelium and cells of the decidua capsularis. It will be noted in the Table that both fetal and maternal parts showed luteotrophic activity; but the maternal part of a 12-day rat placenta weighs approximately 40-45 mg in contrast to 10-15 mg for the fetal part. Further experiments with 10- and 15-day placentae supplemented by estrone proved unsuccessful except in a single instance in which 2 fifteen day placentae (500 mg) were administered with estrone.

*Discussion.* The above data support the earlier finding(1) of a luteotrophic substance in the rat placenta<sup>§</sup> and place the initiation of its secretion at mid-pregnancy. In the Astwood and Greep investigation the question of whether or not estrin formation was induced in the ovary was not raised, probably since allegedly pure progesterone alone suffices to cause decidioma formation in the traumatized uterus. In our previous experiments(4,5) the importance of the synergism between estrin and progestin in the maintenance of pregnancy has been emphasized; and the present investigation brings further proof of that synergism. Finding that a single 12-day placenta weighing 55 mg plus 0.5  $\mu\text{g}$  of estrone accomplished the same result as five 12-day placentae or 275 mg suggested that the equivalent of 0.5  $\mu\text{g}$  of estrone is present in the extra 4 placentae (220 mg) or else a substance capable of inducing the rats' ovaries to form this amount of estrogen may be formed there. Another possibility would be that the 12-day rat placenta contributes some of the necessary estrin and secretes a gonadotrophin capable of causing the ovaries to contribute the rest. The same argument might be used in support of a combined secretion of progestin by ovaries and placenta.

To have proven that the pituitary's gonadotrophic triad, FSH, ICSH and lactogenic hormone may be completely replaced by the placenta during the critical 6-12-day period of gestation in no way leads to the implication that these gonadotrophins are not responsible for the maintenance of the conceptus until mid-term. But it does allow one to surmise that in its earliest existence the rat placenta probably begins to supplement the functions of at least these three pituitary hormones so that by mid-term it may com-

<sup>§</sup> Astwood and Greep were unable to detect other than luteotrophic activity in the rat placentae. We have been able to elicit positive local tests in squabs' crops with acid-acetone extracts of 12-day rat placentae in doses as low as 2 mg equivalents of wet tissue. However, this is an extremely high dose as compared to 0.001  $\mu\text{g}$  of purified lactogenic hormone required for a positive reaction.

5. Lyons, W. R., PROC. SOC. EXP. BIOL. AND MED., 1943, v54, 65.

pletely dispense with that gland.

*Summary.* Pregnancy has been maintained in rats hypophysectomized on day 6 of their first pregnancy and injected daily subcutaneously for 6 days with placentae from rats, 12 days pregnant. Five placentae or approximately 275 mg daily accomplished this in the

majority of the test rats. One 12-day placenta plus 0.5 µg of estrone proved equally efficacious. Pregnancy was not maintained by similar treatment if the rats were also oöphorectomized.

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## Capillary Fragility and Vitamin 'P' Protective Action Against Radiation. (18081)

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(Introduced by Walter H. Eddy)

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When Armentano and associates(1) announced their discovery of a new vitamin 'P', or "permeability vitamin," they did not define what they understood under the term of increased capillary permeability. The absence of any specification concerning vitamin 'P' activity became a source of considerable confusion, which was further increased by the theory of Javillier and Lavollay(2) and Haley *et al.*(3) that increased capillary permeability and fragility are phenomena of a purely neurogenic nature. Yet neither of these investigators made a distinction between the terms of increased capillary permeability and capillary fragility. Haley *et al.*, using a mammalian capillary bed (a rat meso-appendix preparation) for the estimation of the activity of vitamin 'P', measured this activity by visible vasomotion of the arterioles and metarterioles and concluded that only catechins among the flavonoids possess "permeability activity" interpreted in terms of vasomotor response.

The purpose of our present investigation is to show that the flavonoids described by Szent-Gyorgyi and others under the name

of vitamin 'P' exert their effectiveness on the capillary wall mostly if not exclusively in cases when increased capillary fragility is present. We define the term "increased capillary fragility" as a condition when chemical lesions in the capillary wall are apparent, while increased capillary permeability of a temporary nature might occur due to neurogenic factors. In most cases of hemorrhagic diathesis either due to toxic condition or in radiation injury chemical changes in the capillary wall may be detected microchemically(4).

*Experimental.* A vitamin 'P' compound, isolated from citrus fruit and containing 4 identified flavonoid substances† was tested for capillary permeability activity. In the first series of experiments we used Menkin's method(5). He found that an inflammatory exudate induces an increase in capillary permeability, manifested by the accumulation of trypan blue from the circulation in a cutaneous area previously injected with an exudate. This effect was duplicated and even accentuated by leukotaxine, a nitrogenous substance extracted from inflammatory exudation. Ac-

\* Carl T. Tucker Fellow.

1. Armentano, L., Bentsath, A., Beres, T., Rusznyak, B., and Szent-Gyorgyi, A., *Deut. med. Wochschr.*, 1936, v62, 1326.

2. Javillier, M., and Lavollay, J., *Helvetica Chim. Acta.*, 1946, v29, 1283.

3. Haley, T., Clark, W., and Geissman, T., *PROC. Soc. EXP. BIOL. AND MED.*, 1947, v65, 202.

4. Sokoloff, B., and Redd, J. B., "Capillary Permeability and Fragility" Monograph, Florida Southern College, 1949.

† Four factors present in this compound were identified as chalcone hesperidin, glucose-hesperidin, eriodictin and quercitrin-like substance. This compound is similar but not identical to the original "citrin" of Szent-Gyorgyi.

5. Menkin, V., *Am. J. Physiol.*, 1940, v129, 691.

TABLE I. Dye Penetration Rates.

Control rabbits, time of dye penetration in sec:
180, 120, 195, 103, 110, 90, 60, 80, 90, 135 (avg 1 min. 57 sec.)
Vit. 'P' rabbits, time of dye penetration in min.
23, 60, 48, 22, 51, 46, 47, 41, 24, 52, 39, 38, 38, 44, 59, 43, 44, 45, 46, 48 (avg 42 min. 54 sec.)

cording to Menkin its action can be inhibited by an extract of adrenal cortex.

Rabbits averaging 2 kilo in weight were given 10 mg per kilo of weight of a flavonoid substance 20 minutes before injection of 3 mg of leukotaxine. Immediately afterward 12 cc of 1% trypan blue in saline solution was introduced into the marginal ear vein. The capillary permeability was gauged by the degree of dye accumulation in the various skin areas. We found that the citrus vitamin 'P' compound when given in this amount wholly inhibited the effect of leukotaxine of increasing capillary permeability. The citrus vitamin 'P' compound was effective in inhibiting the injurious effect of leukotaxine whether it was injected separately or mixed together. Thus it exerted an effect on capillary permeability similar to the cortical extract of Menkin's experiments.

In our next series of experiments, using the technic of Ambrose and DeEds(6) which consists in the application of chloroform to the skin by means of a cotton-tipped applicator, we tested the citrus vitamin 'P' compound. A dose of 10 mg per kilo of weight was injected subcutaneously 20 minutes before the injection of trypan blue (10 cc) in the marginal ear vein and the application of chloroform wheal to the skin. The average time of the appearance of the dye for 20 tests was 43 minutes as against about 2 minutes in the control batch of 10 rabbits (Table I).

These 2 tests, although reliable for a qualitative testing of vitamin 'P' activity, are not well adapted for quantitative bio-assaying of the potency of these compounds. In search for such a test we began experimentation with a bacterial polysaccharide. The polysaccharide isolated by Shear(7) from *Serratia mar-*

*cescens* produces an extensive hemorrhage in the tumors of animals and, when the dose is sufficiently large, can even cause death. Normal rats and mice are much less susceptible to the toxic effect of this compound and can survive a much larger dose than can the cancerous animals. Four or 5 hours after the bacterial polysaccharide is injected into the cancerous animals, profuse hemorrhages are observed in the adrenal gland. For our experiments we used August Rat Carcinoma, a fast growing tumor. When a rat bearer of a large tumor is given 0.3 mg per 100 g of weight of P-25, a bacterial polysaccharide which we received from Dr. Shear, death occurs in about 7 hours preceded by profuse hemorrhages in the adrenal gland. If a small dose, 3 mg per 100 g of weight, of the citrus vitamin P compound is given subcutaneously, one or two hours before the injection of polysaccharide, the life of the animal is prolonged up to 18-24 hours. A larger dose of the same vitamin 'P' compound given twice, before and shortly after the injection of P-25, will prevent the hemorrhages not only in the adrenal gland but also in the tumor itself. We were able to estimate with a considerable degree of accuracy the exact dose of vitamin 'P' compound which inhibits the effect of polysaccharide on the capillary system. Five mg per 100 g of weight of vitamin 'P' compound, given twice, is the protective dose against the near-lethal dose of polysaccharide. The hemorrhages in the tumor and in the adrenal gland do not occur immediately after the injection of polysaccharide but develop gradually as the result of increasing capillary fragility in the affected area. Apparently this compound causes an injury to the capillary wall in a similar if not identical manner as leukotaxine.

In our next series of experiments, radiation injury to the capillary wall was investigated. One hundred rats were submitted to a total

6. Ambrose, A. M. and DeEds, F. L., *J. Pharm. Exp. Therap.*, 1947, v90, 359.

7. Shear, M. J., *J. Nat. Cancer Inst.*, 1943, v4, 81.

## CAPILLARY FRAGILITY AND VIT. 'P' PROTECTIVE ACTION

TABLE II. Control Groups (40 rats).

No. of days of survival	11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23
No. of rats succumbed	1, 3, 3, 2, 3, 3, 3, 3, 2, 4, 2, 1, 2 = 32
Mortality rate:	80%

TABLE III. Rats Given 4 mg of Vit. 'P' for 10 Days (20 rats).

No. of days of survival	17, 18, 19, 20, 21, 22, 23, 24, 25
No. of rats succumbed	1, 1, 2, 0, 0, 1, 1, 1, 1 = 8
Mortality rate:	40%

TABLE IV. Rats Given 5 mg of Vit. 'P' for 30 Days (40 rats).

No. of days of survival	18, 19, 20, 21, 22, 23, 24, 25, 26
No. of rats succumbed	1, 0, 0, 0, 1, 0, 0, 1, 1 = 4
Mortality rate:	10%

body, near-lethal dose of radiation. The radiation factors were: 250 kv, with 0.5 mm Cu and 3.0 mm Bakelite Filters. Target distance was 27.5 cm and 210 r/min dose rate. All rats received 800 r total body X radiation in a single exposure. Forty rats served as control. The mortality rate in the control group was 80% (Table II). All survivors manifested gross hemorrhages of various degrees of gravity. Of the treated animals 20 were given 4 mg of citrus vitamin 'P' for 10 days, 3 days prior to radiation and 7 days post radiation. This dose gave a partial protection. The mortality rate was reduced to 40% (Table III). Forty rats were given 5 mg of vitamin 'P' compound for 30 days, with a total amount of 150 mg. In this group the mortality rate was reduced to 10% (Table IV).

The results of this series of experiments seem to indicate that the flavonoid compound, vitamin 'P', gives considerable if not complete protection against a total body near-lethal dose of radiation. The absence of hemorrhages and the state of the capillary wall of the treated animals suggests that this protective action of flavonoids concerns chiefly if not exclusively the capillary system. The striking fact is that injury to the capillary wall, whether it is caused by ionizing radiation or by leukotaxine, or by a bacterial polysaccharide, can be prevented to a considerable degree by the administration of the same flavonoid compound.

*Discussion.* All the available evidence indicates that the capillary wall in normal tissue is comparatively impermeable both to

serum albumin and to serum globulin. Therefore, the average effective pore size of the capillary wall must be less than 6 millimicrons while the spaces between adjacent endothelial cells are greater than 15 millimicrons. It is established that pore size is defined by the intercellular cement lying between the endothelial cells. According to the work of Chambers and Zweifach(8) and others the cement dissolves under certain physical conditions such as the absence of calcium or at slightly acid pH. Danielli and Stock(9) pointed out: "It is quite certain that anything which affects the physical condition of the intercellular cement affects the pore size." This in turn will affect the effectiveness of the pores as "perfect sieves" and consequently induce what we know as increased capillary fragility. According to Danielli and Stock "under normal conditions the walls of the pores through this cement are coated with a layer of adsorbed serum protein which effectively reduces the area of pore through which filtration may occur." Displacement of this adsorbed serum protein by other molecules of small diameter may result in an increase in pore size and in a consequent injury to the capillary wall. In toxic conditions when increased capillary fragility is manifested such displacement might take place. Although Duran-Reynals(10) sug-

8. Chambers, R. and Zweifach, B. M., *J. Cell. Physiol.*, 1940, v15, 255.

9. Danielli, J. F. and Stock, A., *J. Physiol.*, 1945, v110, 81.

10. Duran-Reynals, F., *Yale J. Biol. Med.*, 1939, v11, 601.

gested that the "spreading factor," hyaluronidase causes an increase in capillary fragility by affecting the intercellular cement, other investigators were unable to confirm this observation. Danielli and Stock have stated that in all probability the injury to the capillary wall in toxic conditions is direct and not due to enzymic action.

It is even more unlikely that the "spreading factor" plays a role in the course of radiation injury. This impression is supported by the failure in the work of Rekers and Field(11) on dogs of such potent hyaluronidase inhibitors as dopa and sodium gentizate to influence the course of radiation. These results showed that the effect of vitamin 'P' factors seems also to be directly on the intercellular substance. They obtained no evidence that these factors inhibit heparin, for the heparin content was not altered by vitamin 'P' therapy to any great degree.

Rekers and Field also found that certain flavonoids gave considerable protection against a total body near-lethal dosage of radiation. They concluded that "previous misunderstanding of the nature of vitamin 'P'" has arisen from both the failure to recognize that several flavonone analogues possess very sim-

ilar anti-hemorrhagic activity and that ascorbic acid has the capacity to potentiate activity in other flavonones." They are inclined to conclude that vitamin 'P' factors affect the capillary system directly perhaps participating as a principal in the 'wear and tear' of a part or all of the capillary system, inhibiting degeneration and taking part in its regeneration. The results of our present investigation are in full accordance with their viewpoint and give further support to the original work of Armentano *et al.*(1) as to the role which the factors of vitamin 'P' play in increased capillary permeability. This term should be interpreted, however, as indicative of chemical lesions in the capillary wall, or more exactly as increased capillary fragility.

*Conclusion.* Administration of leukotaxine, a bacterial polysaccharide or an exposure to ionizing radiation induces increased capillary fragility. Injury to the capillary wall, whether it is caused by ionizing radiation, by leukotaxine, or by a bacterial polysaccharide, can be prevented to a considerable degree by the administration of a vitamin 'P' compound composed of flavonoids naturally present in citrus fruit.

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11. Rekers, F. E. and Field, J. B., *J. Clin. Invest.*, 1949, v28, 746.

## Occurrence of Endocarditis with Valvular Deformities in Dogs with Arteriovenous Fistulae.\* (18082)

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The accidental finding of endocarditis in dogs has been reported to be extremely rare (1). Likewise the dog is generally believed to be a relatively resistant animal to the

experimental production of endocarditis(2). It is therefore of interest that the application of an increased work load of a particular type to the canine heart was followed by a high incidence of endocardial vegetations without the necessity of intentional injection of any bacteria. The observations herein reported were made incidental to a study of heart failure in the dog induced by an increase in work load. This increase in work load of the heart has been accomplished by large

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1. Morehead, R. P. and Little, J. M., *Am. J. Path.*, 1945, v21, 339.

2. Clawson, B. J., Personal Communication, 1949.

arteriovenous fistulae made surgically under aseptic conditions. Where more than one fistula was made in a particular dog, the operations were staged one to 4 weeks apart. Following the construction of arterio-venous fistulae of sufficient size, it was necessary only to allow a minimum time of approximately 4 to 6 weeks to elapse for the development of endocarditis in the animals in which physiological studies as outlined below were carried out.

*Method of study.* Mongrel dogs previously dewormed and of either sex were used in these studies. All animals were allowed a period of at least 2 months in the animal colony for purposes of acclimatization before they were utilized in these experiments. Animals not in good health for any reason at the end of this period of time were not operated upon. Dogs of two estimated age groups were used. The criteria for estimating age have been described(3). Group 1. Young dogs—2 to 5 yrs. Group 2. Old dogs—10 or more yrs. All dogs not dying during the course of these experiments were sacrificed by intravenous pentobarbital. An autopsy was performed on all animals at the time of death.

*Operative procedure.* The iliac fistulae were made immediately distal to the trifurcation of the abdominal aorta between the iliac artery and vein. The femoral fistulae were made 2 to 4 cm below the inguinal ligament between the femoral artery and vein. The aorta-vena cava anastomoses were made just proximal to the trifurcation of the abdominal aorta. In all cases the artery-vein anastomoses were made side to side using a single running stitch of fine silk (6-0) onatraumatic needles. The lengths of all fistulae were measured at the conclusion of the anastomosis after release of the blood vessel clamps and again at autopsy. With only one exception (Dog 36)<sup>§</sup> all iliac and femoral fistulae were made 23 to 40 mm in length. Since in all of these cases the diameter of the fistula substantially exceeds that of the parent artery these

minor differences in the length of the fistula can probably be disregarded in making comparisons. All animals were given prophylactic injections of penicillin in oil 150,000 units daily for one week following each operative procedure.

*Diagnosis of endocarditis.* The term endocarditis is used in these studies to denote an inflammation of the lining of the heart especially that overlying the valves. The diagnosis of endocarditis in these dogs has been based upon the following criteria:

1. Development of fever.
2. Development of elevated blood sedimentation rate.
3. Development of heart murmurs, systolic and diastolic.
4. Observation of petechial phenomena (in eyes, lungs, kidneys).
5. Recovery of organisms from the blood.
6. Observation of typical gross and microscopic autopsy findings within the heart.

*Results.* The incidence of endocarditis in all animals with arteriovenous fistulae studied is indicated in Tables I, II and III. Inspection of these tables indicates several factors of probable importance in the development of endocarditis. These factors will be discussed under the following headings: (1) relationship of the arteriovenous fistula load to the incidence of endocarditis, (2) duration of the fistula load, (3) age of the animal, (4) pathology, (5) bacteriology, and (6) adrenal weights.

*1. Relationship of arteriovenous fistula load to incidence of endocarditis.* We observed in these studies that there is a very definite level to which one must increase cardiovascular stress in order to be followed regularly by the development of endocarditis. With degrees of cardiovascular stress less than this level the hearts remained immune to the development of "spontaneous" endocarditis. These relationships between degree of increased fistula load and the occurrence of endocarditis observed in these studies are indicated in Table IV.

There are several measurable factors which influence the degree of cardiovascular stress created by an arteriovenous fistula. (a) The diameter of fistula. (b) The position of fistula. As stated above, all of these animals may be considered to have arteriovenous fis-

3. Lillehei, C. W. and Wangensteen, O. H., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 129.

§ Iliac fistula = 15 mm.

TABLE I. Dogs with Large\* Arteriovenous Fistula Load. Young age group (2 to 5 yr).

Dog No.	Arteriovenous fistula		Days survival after		Cause of death	
	No.	Location	Fist. I	Fist. II		
A. With endocarditis	114	2	Iliac-femoral	42	30	Endocarditis
	250	2	Iliac + aorta-vena cava	98	60	"
	124	2	Iliac-femoral	109	76	Heart failure (induced with NaCl)
	451	2	Iliac-iliac	112	27	Endocarditis
	26	2	Iliac-femoral	120	35	"
B. Without endocarditis	35	2	" "	121	63	"
	24	2	" "	148	137	"
	110	1	Aorta-vena cava	2	—	Acute heart failure
	47	2	Iliac-iliac	31	1	Died postop.
	69	2	Iliac-femoral	48	41	Heart failure
	49	3	" "	49	21	" " †
			aorta-vena cava	2 (3rd fistula)		
	431	2	Iliac-femoral	61	20	Sacrificed§
	71	2	" "	109	81	Heart failure
	130	2	Iliac-iliac	276	33	Sacrificed‡

\* Fistula load sufficient for the development of endocarditis.

† Iliac fistula partially closed prior to making aorta-cava fistula.

‡ Dog died 2 days after the 3rd (aorta-vena cava) fistula made, other fistulas partially closed.

§ Sacrificed to obtain weight of adrenal glands.

TABLE II. Dogs with Large\* Arteriovenous Fistula Load. Old age group (10 yr old or more).

Dog No.	Arteriovenous fistula		Days survival after		Cause of death	
	No.	Location	Fist. I	Fist. II		
A. With endocarditis	6	1	Iliac	55	—	Endocarditis
	37	2	Femoral-femoral	81	69	"
	36	2	Iliac-femoral	109	87	Sacrificed†
B. Without endocarditis	305	1	Iliac	1	—	Acute cardiac failure
	17	1	"	13	—	Gangrene of leg
	4	1	"	36	—	Progressive heart failure

\* Fistula load sufficient for the development of endocarditis.

† Endocarditis cured with aureomycin 2 mo. before sacrificed.

TABLE III. Dogs with Small\* Arteriovenous Fistula Load. Control series (young age group, 2-5 yr).

Dog No.	Arteriovenous fistula		Days survival after		Result	Cause of death
	No.	Location	Fist. I	Fist. II		
34	1	Femoral	30	—	No endocarditis	Sacrificed
70	1	Iliac	115	—	Living	No evidence of endocarditis
62	1	"	124	—	"	"
100	1	"	125	—	"	"
56	1	"	126	—	"	"
59	1	"	202	—	"	"
135	1	"	237	—	No endocarditis	Sacrificed
79	1	"	289	—	"	"
591	2	Femoral-femoral	355	350	"	"

\* Dogs with arteriovenous fistula load insufficient for the occurrence of endocarditis.

tulae of the same approximate length. The position of the arteriovenous fistula appears to be of importance. The larger the vessels in the lower extremity in which the fistulae were produced the greater was the incidence

of endocarditis. The fistulae in the larger vessels obviously have the larger blood-shunting sections.

2. Duration of the fistula load. The duration of the arteriovenous fistula is of some

TABLE IV. Relationship of Arteriovenous Fistula Load and Endocarditis.

Dog age (yr)	Sufficient for endocarditis	Not sufficient for endocarditis
Young (2-5)	One iliac + , femoral, or 2 iliac, or aorta-cava A.V.F.	One iliac, or 2 femoral A.V.F.
Old (10 or more)	One iliac, or 2 femoral A.V.F.	1 femoral A.V.F.

importance. Following the introduction of an arteriovenous fistula load sufficient<sup>||</sup> to be followed by endocarditis the earliest that we have been able to diagnose endocarditis is approximately one month. Moreover, in the group of 10 animals (Tables I and II) with the larger arteriovenous fistula loads, which were sufficient in size to result in the spontaneous development of endocarditis, all had died within 148 days. In the ten other animals (Tables I and II) with sufficient fistula loads but which did not develop endocarditis, the duration of life was significantly shorter, although there are several exceptions. It was necessary also to sacrifice several of these dogs with large arteriovenous fistula loads at a time when they did not have endocarditis in order to obtain information as to the relationship of adrenal hypertrophy to valvulitis. It is possible that they also would have developed endocarditis had they been allowed to survive longer. On the other hand in the nine dogs with an insufficient load (Table III), none has developed endocarditis although we have observed them as long as 355 days under exactly similar conditions. Thus, it is apparent that the size, position, and duration of the fistulae are all important determining factors for the total arteriovenous fistula load on the organism.

3. *Age of the animal.* In these experiments the age of the animal was important only in regard to the position of the fistulae. In old dogs bilateral femoral fistulae or a single iliac fistula resulted in endocarditis, whereas larger vessels needed to be involved in young

dogs in order to be followed by endocarditis. However, by adjusting the size, position, and number of arteriovenous fistulae it has been possible to observe endocarditis in an equally high incidence in all age groups.

4. *Pathology.*<sup>¶</sup> Grossly, the valvular lesions varied in appearance from the soft friable vegetation morphologically similar to those seen in bacterial endocarditis in man to the firm smooth vegetations typical of rheumatic endocarditis (Fig. 1 and 2). Damage to the point of actual rupture of the valve cusp has occurred frequently. Likewise, microscopically the pathological changes in the heart valves show striking similarities to bacterial and to rheumatic endocarditis as seen in man. Often one leaflet may show only bacterial vegetations while another within the same heart shows only the rheumatic type. And occasionally these two types of lesions were seen within different parts of the same leaflet. Vegetations were also found sometimes on the mural endocardium. All valves have been involved except the pulmonary, although not all valves were involved in every animal. Of the ten dogs which developed endocarditis during this study, at the time of death three had vegetations at the site of their arteriovenous fistulae while in the remainder the fistulae were clean. Hemorrhage into valves sometimes occurred. These pathological findings in the heart are summarized in Table V.

One animal in this series with endocarditis (Dog No. 114) has been found to have a typical microscopic picture of acute proliferative glomerulonephritis\*\* in the kidneys.

5. *Bacteriology.* In all but the earliest animals studied repeated blood cultures have been made.<sup>††</sup> These findings are summarized in Table V. In all dogs with endocarditis whose blood was cultured, organisms have

<sup>¶</sup> A morphological study of these materials is nearly complete and will be published in cooperation with Professor B. J. Clawson, Department of Pathology, University of Minnesota.

<sup>\*\*</sup> Verified by Professor E. T. Bell, Department of Pathology, University of Minnesota.

<sup>††</sup> With the cooperation of Professor Wesley W. Spink, Department of Medicine, University of Minnesota.

|| Refer to Table IV for definition of terms "sufficient" and "insufficient" as used in context in reference to arteriovenous fistula load.

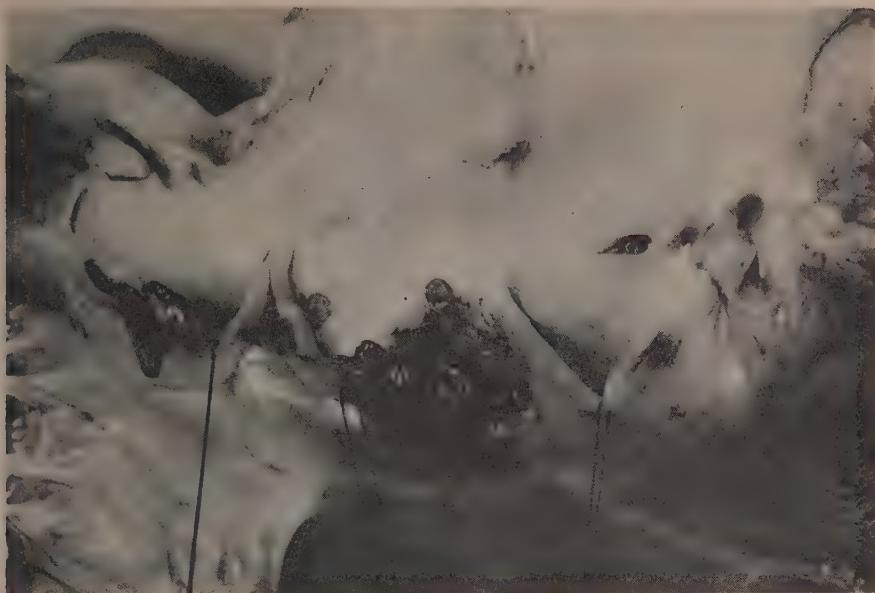


FIG. 1.

Aortic valve, Dog #35. (Right iliac and left femoral arteriovenous fistulas). Note: Soft friable vegetations typical of bacterial endocarditis.



FIG. 2.

Mitral valve, Dog #6. (Old dog with single arteriovenous fistula). Note: Firm smooth vegetations resembling rheumatic endocarditis, and thickened chorda tendinae.

TABLE V. Valve Involvement and Infecting Organism in Endocarditis.

Dog No.	Age	A.V.F.			Mitr.I	Tricusp.	Organism
		No.	Location	Aortic			
451	Y.g.	2	I,I	0	+	0	Not done
114	"	2	I,F	+	+	+	<i>Strep. viridans</i>
26	"	2	I,F	+	0	0	<i>Aerob. aerogenes</i>
250	"	1	A-Vc	0	+	0	Not done
35	"	2	I,F	+	+	0	<i>Aerob. aerogenes</i>
124	"	2	I,F	0	0	+	Not done
24	"	2	I,F	0	+	+	Hemolytic strep.
36*	Old	2	I,F	0	+	0	<i>Aerob. aerogenes</i>
6	"	1	I	+	+	+	Not done
37	"	2	F,F	0	+	0	<i>Aerob. aerogenes</i>
Total		4		8	4		

\* Blood cultures became negative and clinical condition indicated abatement of the active infection after aureomycin therapy.

I = Iliac A.V.F.

F = Femoral A.V.F.

A-Vc = Aorta-vena cava A.V.F.

been present in the blood. It has been of interest to us, however, that there appears to be nothing specific about the type of organism necessary to cause endocarditis nor does there appear to be any relationship between the type of infecting organism and the type of lesion within the heart, i.e. rheumatic-like or bacterial-like. However, it may be significant that the animal mentioned above which developed glomerulonephritis was the only animal in the series with a *Streptococcus viridans* septicemia. Upon histologic examination bacteria were found in the valve lesions.

6. Adrenal weights. It was noted early in the course of these studies that the animals which developed endocarditis had large adrenal glands. In all animals studied the adrenal glands have been weighed to the nearest milligram on an analytical balance soon after death. Results of these observations are denoted in Fig. 3. From these data it is apparent that endocarditis was associated with heavier than normal adrenal gland weights and also that this increase in adrenal

gland weight preceded the occurrence of endocarditis. Further, those animals with arteriovenous fistulae insufficient to cause endocarditis appear to have adrenal glands of normal weight.

*Discussion.* From the data presented it is apparent that under the conditions of this experiment dogs "spontaneously" developed a high incidence of severe endocarditis which progressed usually to death. This sequence of events differs materially from the type of experimental endocarditis which has been reported in the past(4-8). In the usual types of experimental endocarditis the infections have been produced by injection of large numbers of often supposedly specific organisms with or without some kind of mechanical damage to the heart valve. This type of endocarditis has usually been characterized by a lower incidence of animals infected, relatively less severe lesions within the heart, and a marked tendency for the endocarditis to heal.

The dogs in this study were subjected to various physiological measurements. In addition to the stress of the arteriovenous fistulae some of the animals were subjected to extra sodium chloride and/or work on the treadmill. However, not all of the endocarditis dogs were subject to either of the above added stresses. All but 2 of the dogs developing endocarditis had physiological studies performed upon them involving venapuncture. Of the animals with fistulas of smaller vessels, showing no endocarditis, all but one had comparable physiological studies. In all probability organisms were introduced adventitiously by these maneuvers which were performed with clean but not aseptic technics. However, the fact that the animals with the lesser cardiovascular stresses imposed showed no endocarditis, seems to indicate that the primary factor in the susceptibility to endocardial infection is the cardiovascular stress.

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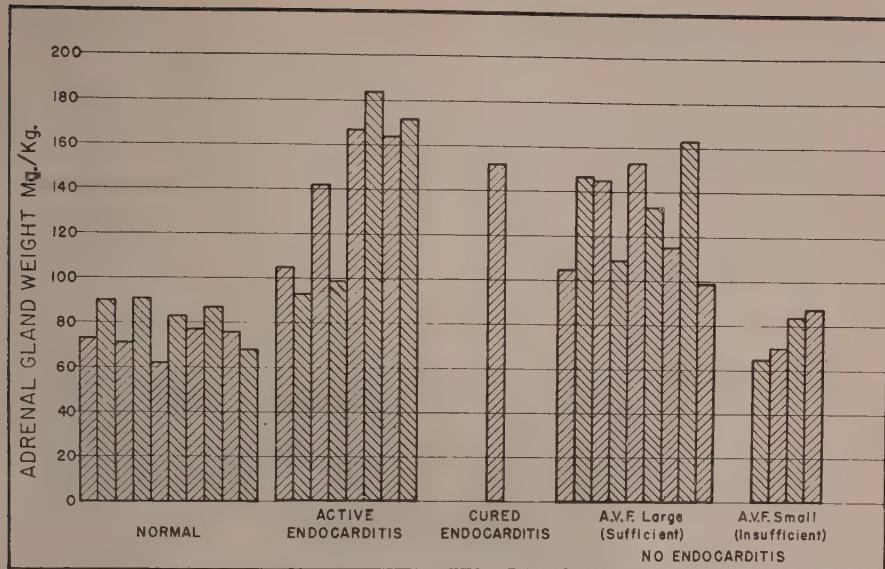


FIG. 3.

## Relationship of Adrenal Gland Weight and Endocarditis.

The dogs dying or sacrificed with active endocarditis had heavier than normal adrenal glands. Also animals with large arteriovenous fistulas sufficient in size\* to be followed by endocarditis had adrenal enlargement when sacrificed prior to the development of endocarditis. This adrenal enlargement was not seen in animals with smaller\* arteriovenous fistulas in whom there was no increased susceptibility to endocarditis.

Mechanical trauma to the valves associated with the increased cardiac output may be a conditioning factor.

An arteriovenous fistula produces a lowering in the total peripheral resistance, the compensation for which is an increase in cardiac output(9). In our studies the cardiac outputs were elevated as much as six-fold, as measured by the direct Fick procedure. The cardiac silhouette as measured by planimetry from 6" plates was increased. The plasma volume and total extracellular fluid space determinations also showed great elevations, the former up to 100% and the latter by as much as 30%. These observations confirm earlier studies. A large fistula also produces a corresponding rise in pulmonary artery pressure. The average value of the mean integrated pulmonary artery pressure measured with a Statham strain gauge was  $16.2 \pm 1.1$  mm Hg in the normal state as

compared with  $31.3 \pm 1.4$  mm Hg in the same animals after creation of the fistulae in dogs developing endocarditis. The mean carotid artery pressure as measured by mercury manometer was not significantly altered by a fistula. Detailed reports on these observations will be made later.

The present study does not indicate what changes as outlined above may be determining in the results observed. However, the increased mechanical work of the heart is obviously a central factor. The question is by what intimate mechanisms this change might influence the ultimate production of endocarditis. One observed fact, namely the increased adrenal weight at death in dogs developing endocarditis, is of interest in this regard. The dogs sacrificed early after bilateral fistula production and without endocarditis showed comparable large glands, indicating that the enlargement precedes the development of valvulitis. It would not serve a useful purpose to speculate at this time about the mechanism by which the stress, the en-

\* See text for definition of size.

9. Holman, E., Arteriovenous Aneurysm. The Macmillan Co., New York, 1937.

docarditis and the adrenal hypertrophy are interrelated. Further studies of this relationship are in progress.

The occurrence, in one dog, of a typical glomerulonephritis associated with endocarditis is perhaps of significance in the light of the frequent clinical association of these two entities.

**Summary.** Observations have been reported in which after the creation of large

arteriovenous fistulae in dogs endocarditis occurred without intentional introduction of bacteria. This result occurred in about 8 out of 10 dogs in which sufficiently large shunts existed for more than 4 weeks. Adrenal gland enlargement occurred following creation of a large fistula. Other concomitant findings have been reported and discussed.

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### Plasma and Blood Volumes of Mouse Organs, As Determined with Radioactive Iodoproteins.\* (18083)

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In connection with the investigations of the localization of radioactive antibodies in various tissues as carried out in this laboratory (1-4), it was necessary to know the plasma and blood volumes of several organs of the mouse. These volumes were determined by injecting mice with radioiodinated proteins and assaying the organs for their radioactivity content. The results are reported here(4a). Similar use of radioiodinated proteins has been made by Fine and Seligman(5) and Gibson *et al.*(6-8) in studies on plasma vol-

umes in traumatic shock in dogs.

We used 3 different preparations of radioiodinated protein. These were the globulin fraction of rabbit antiovalbumin serum, and 2 preparations of bovine serum albumin which were iodinated with iodine containing radioactive  $I^{131}$ , according to a method described previously(1). The bovine serum albumin was the crystallized product obtained from the Armour Co., Chicago. The globulin fraction of the antiovalbumin serum was the same preparation described previously(4). The mice used were 6 to 7 week old males, weighing 17 to 25 g, from the inbred Akm strain. The hematocrit was determined on the heparinized blood of 3 males weighing 19 to 20 g. These animals had received an intravenous injection of 0.1 ml of a heparin solution 3 minutes before drawing the blood. Two samples, one each from the caudal and jugular

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† Senior Fellow in Cancer Research, American Cancer Society Fellowship recommended by the Committee on Growth of the National Research Council.

‡ Visiting Fellow, Sloan-Kettering Institute, Jan.-July, 1948.

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§ The radioactive iodine was obtained from the U. S. Atomic Energy Commission, Oak Ridge Operations, Isotopes Division, Oak Ridge, Tenn.

veins of each mouse, were taken in Van Allen hematocrit tubes. These were centrifuged for 30 minutes with a force of 1000 times gravity. The mean hematocrit was  $44.6 \pm 0.5$ . For determination of the plasma and blood volumes, the animals were injected intravenously with the radio-protein containing about  $0.5 \mu\text{c}$  of radioiodine after they had been kept in an incubator at  $37^\circ\text{C}$  for a short while (at least 15 minutes) to produce peripheral vaso-dilatation which facilitated injection. The radio-protein was allowed to circulate for at least 15 minutes (and as high as one hour) in order to obtain complete mixing and to permit the animal to equilibrate with the ambient room temperature. In the meantime the total radioactivity in the mouse was determined, by measuring the gamma ray activity, by placing the live mouse in a tube surrounded by a multiple section gamma ray counter according to the method described previously (2). The animal was chloroformed, the tail cut off and a measured volume of blood was obtained from the stump. The tail was immediately ligated and the total volume of blood drawn was kept less than  $100 \text{ mm}^3$  to minimize alteration in the blood volume of the animal. The specific activity of the measured sample, as determined by beta ray count as described below, was used as the base for calculating plasma volumes in the organs. In the first six animals assayed, the volume of the blood sample was determined with a 0.25 ml tuberculin syringe. For the other 3 animals, a  $20 \text{ mm}^3$  Sahli pipette was used. The animal was then killed with chloroform and opened ventrally. The blood vessels of each organ to be assayed were ligated with nylon thread as close to the organ as possible to prevent loss of blood, and the organs were then excised. Ligating was not feasible for the small intestine, long bones of the hind legs and their associated muscles, and the brain. These organs were, therefore, excised last on the assumption that sufficient stasis would set in to prevent any significant loss of blood.

After excision, the organs were weighed and then the beta ray count determined according to the method described previously (2). The organs were homogenized and the

TABLE I. Wet Weight of Mouse Organs.

	Mean wt in mg	% of total body wt	
		Mean & S.E.	Range
Whole animal*	22,200	$1.74 \pm .08$	1.47-2.25
Brain†	380	$1.70 \pm .07$	1.42-2.04
Kidneys*	375	$7.6 \pm .2$	6.8-8.2
Liver‡	1,670	$1.18 \pm .10$	.46-1.53
Long bones‡	265	$.71 \pm .05$	.59-1.02
Lungs§	156	$.37 \pm .05$	.12-.56
Spleen†	85	$.56 \pm .06$	.23-.74
Sub-maxillary glands§	125	$.49 \pm .04$	.33-.82
Testes*	110		

\* Avg values for 9 Akm male mice of 17.4 to 25.2 g.

† 8 mice.

‡ Femur, tibia, and fibula of both hind legs.

§ 7 mice.

homogenate made alkaline with sodium hydroxide. The homogenate was poured into metal caps, evaporated to dryness under infra-red lamps, and each cap then assayed for its radioactivity count with a thin window G.M. tube. Where the total wet weight of an organ was over 500 mg (which would result in a sample thicker than about  $10 \text{ mg per cm}^2$  for a single cap), the homogenate was poured into several caps, and the total of the counts for the caps was determined. The plasma volumes of the organs were determined as the ratio of the radioactivity per unit weight of the organ to the radioactivity per unit volume of plasma. The radioactivity per unit volume of plasma was calculated from the hematocrit and the radioactivity count of the blood sample from the tail. The plasma volume of the whole mouse was determined from the dilution of the injected radioactivity. The injected radioactivity was established from the gamma ray count for the whole mouse, as described above. The blood volume for the various organs and the whole mouse were calculated from the plasma volumes and the hematocrit, obtained as described above. No correction was made for the probable difference in the hematocrits for the various organs.

The data are presented in Tables I through IV. In Table I are listed the mean wet weights of the various organs and the average percentages of total body weight which each organ comprised (with the standard

## BLOOD VOLUMES OF MOUSE ORGANS

TABLE II. Injection Protocol for Radioiodoprotein.

Animal No.	1	2	3	4	5	6	7	8	9
Animal wt (g)	17.4	25.2	19.6	21.8	23.7	21.3	23.0	24.5	23.3
Type of protein inj.	Anti-ovalbumin serum	Preparation 1			Radioiodinated bovine serum albumin				
Amt of protein inj. (mg)		2.3	3.8	0.4	0.7	0.8	1.3	2.6	5.0
Amt of I <sup>131</sup> inj. ( $\mu$ c)	.063	.071	.066	.048	.049	.055	.048	.114	.050
Vol. of fluid inj. (mm <sup>3</sup> )	500	150	250	20	35	40	65	130	250
Time between inj. of protein and death (min.)	15	60	15	15	15	15	20	20	15

TABLE III. Mean Plasma and Blood Vol. of Mouse Organs in ml per 100 g of Wet Tissue.

	Plasma volume		Mean blood vol.*	% distribution of total plasma
	Mean and S.E.	Range		
Whole animal†	6.7 ± .4	4.6- 8.3	12	100
Brain‡	1.6 ± .1	1.1- 1.8	3	.4
Kidney†	19.1 ± 1.5	13.0-24.2	34	4.8
Liver†	20.2 ± 1.8	12.7-29.4	36	23
Long bones†	6.4 ± .7	3.5- 9.9	11	1.2
Lung§	27.4 ± 2.5	17.9-40	49	2.6
Small intestine†	5.0 ± .6	1.4- 7.5	9	—
Spleen‡	9.2 ± 1.3	5.8-15.8	17	.5
Striated muscle†	1.6 ± .1	.9- 2.1	3	—
Sub-maxillary gland§	5.9 ± .9	3.7-11.1	11	.5
Testes†	3.4 ± .6	1.9- 8.0	6	.2

\* Based upon a hematocrit value of 44.6 ± 0.5 for heparinized blood from the caudal and jugular veins of 3 mice. † Avg for 9 mice. ‡ 8 mice. § 7 mice.

error and range). The averages are for 9 mice weighing from 17.4 to 25.2 g. These organ weights are comparable to the weights in mature mice which have been reported previously by Kopec and Latyszewski(9,10).

Table II gives the injection protocols for each animal. It shows the radioprotein injected, the amounts injected, and the time lapse between the injection of protein and death of the animal.

Table III gives the mean total plasma volume and the mean plasma volumes of the various organs in ml per 100 g of wet weight along with the range of values obtained and the standard error. The mean blood vol-

umes are also given as calculated from the plasma volumes and the hematocrit of the blood from the jugular and caudal veins. The last column gives the percentage distribution of the total plasma among the various organs. In general, there was no significant variation in any of the values determined for the animals of different body weight or when different proteins or protein preparations were used, nor did it appear to matter whether an animal were analyzed either at one hour, or at 15 to 20 minutes, after injection. It was felt that a 15 minute interval was sufficient to insure thorough mixing of the iodinated protein and the blood. Longer circulation times were generally avoided, since Fine and Seligman(5) have shown that in dogs, iodinated bovine serum albumin was removed from the circulation to the extent of 10-25% within the first hour after injection. The effects of such a removal would

9. Kopec, S., and Latyszewski, M., *Memoires de l'Inst. Nat. Polonais d'Economie Rurale a Pulawy*, 1929, v10, 509.

10. Kopec, S., and Latyszewski, M., *Memoires de l'Inst. Nat. Polonais d'Economie Rurale a Pulawy*, 1931, v12, 462.

TABLE IV. Total Blood Volume in the Mouse, as Reported in the Literature(4a).

Authority	Methods used for determining blood vol.	No. and type of mice	Avg blood vol. in ml/100 g body wt
Dreyer and Ray(12)	Mincing and extraction of hemoglobin	19	5.8
Oakley and Warrack(13)	Perfusion and exsanguination	46 ♂ & 54 ♀	6.3
Taylor(14)	Exsanguination and mincing	40 strain dba 16 strain C57	5.23 ± .31 4.9 ± .17
Furth and Sobel(11)	a. Exsanguination and perfusion b. Dye (T-1824) c. Simultaneous dye (T-1824) and exsanguination methods	a. 12 b. 9 c. "Several"	a. 5.2 b. 9.0 c. 5.7 by exsanguination, 10 and 11.7 by dye
Kaliss and Pressman, 1949	Circulating radio-iodoprotein	9 strain Akm	12.1 ± .8

be reflected in an apparent increase in the total plasma volume.

Previous reports of total blood volumes in the mouse are summarized in Table IV. These values vary from 4.9 to 11.7 ml per 100 g of tissue. The single important cause of the wide differences in the values obtained is apparently due to the methods used. This is clearly shown by the data of Furth and Sobel(11) who obtained values by the dye (T-1824) dilution technic which are in agreement with the figures reported here, but are nearly double those obtained by them with perfusion and exsanguination methods. It is most certainly true that the dye technic gives a much closer approximation to a true value than does exsanguination, and that the values for the former technic and those obtained with circulating radio-proteins are in agreement. In a comparison of plasma volumes in dogs, determined by the use of the dye T-1824 and radioiodoalbumin circulating in the plasma, Gibson *et al.*(6) found the values to correspond within ±10%, showing

good agreement between the two methods.

There are several possible reasons for the variations shown from animal to animal in the plasma and blood volume values that were obtained here, such as the "normal" variations that must exist, particularly for the blood vascular system as a whole, which is so sensitively affected by the physiological and nervous inter-relationships of the body; the handling of the animals; the anaesthetics used; the method of killing the animals prior to dissection and the surrounding air temperature. These as well as other factors may influence both the total blood volume, and the blood volumes of the individual organs. The dissection technics can lead to variations in estimates due to possible loss by hemorrhage, particularly from the large blood vessels. This is especially true for the lungs, where it was difficult to tie off all large vessels at a point close to the lungs. This type of error would operate particularly for the brain, small intestine, and striated muscles, where no attempt was made to tie off the blood vessels prior to dissection. A source of discrepancy in estimating the whole blood volume of an organ lies in the differences of hematocrits between the larger blood vessels and the capillaries. This has been well brought out by the studies of Gibson *et al.*(6) on the distribution of plasma and red cells in the large and minute vessels of the dog.

11. Furth, J., and Sobel, H. I., *J. Nat. Cancer Inst.*, 1946, v7, 103.

12. Dreyer, G., and Ray, W., *Roy. Soc. London Philos. Trans.*, 1910, v201B, 133.

13. Oakley, C. L., and Warrack, G. H. *J. Path. Bact.*, 1940, 50, 372.

14. Taylor, A. Univ. of Texas Publication No. 4507 *Cancer Studies*, 1945, p95.

They found hematocrit ratios of 0.85 for the blood of the body, and 0.7 for the minute blood vessels as compared with arterial hematocrits.

Furthermore, the hematocrits differ from organ to organ. Gibson *et al.*(6) found average hematocrit values in the small blood vessels of the dog ranging from 15% for the kidneys to 82% for the spleen. If similar differences hold true for the mouse, as they most certainly must, then estimates of whole blood volume values for a specific organ obtained with the help of hematocrits for venous blood (or arterial blood) must suffer from attendant errors.

**Summary.** The plasma and blood volumes of mice and of various mouse organs were determined by injecting mice with a protein iodinated with iodine containing tracer quantities of radioactive iodine, and determining the amount of radioactivity present in the various organs. The average plasma and blood volumes of the mice were found to be 6.7 ml and 12.7 ml per 100 g of body weight, respectively. The average plasma volume in ml per 100 g of wet tissue for the brain was 1.6; kidney, 19.1; liver, 20.2; lung, 23.9; small intestine, 5.0; spleen, 9.2; submaxillary gland, 5.9; testes, 3.4.

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### Sex Differences in Weight-stimulating Effect of B<sub>12</sub> in Rats on Diets of Varying Composition.\* (18084)

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In this laboratory as elsewhere(1) it has been observed that young rats depleted of B<sub>12</sub>, and receiving either vitamin-free casein or soybean protein as the protein moiety of an adequate diet, show superior weight gains when given daily intramuscular injections of B<sub>12</sub>. This has been noted in the case of both proteins fed at high and low levels. The greater gains were not accompanied by greater nitrogen retention(2).

In an effort to determine whether the source of the calories plays a role in the weight gains achieved with and without B<sub>12</sub>, as indicated by the investigations on the growing mouse by Bosshardt, Paul, and Barnes(3), diets were fed that were high,

low or moderate in fat and/or carbohydrate content. The protein level was so adjusted that the percentage of calories from the protein was approximately the same in each ration.

**Experimental procedure.** The composition of the rations used is given in Table I. Twenty young rats of both sexes, 26 to 30 days old,

TABLE I. Composition of Rations Used.

	A %	B %	C %	D %
Casein (1)	40.	53.5	34.	34.
Salts #4 (2)	4.	4.	4.	4.
Primex (3)	12.	41.2	—	—
Cottonseed oil (4)	1.	1.	1.	1.
Maize dextrin (5)	42.7	—	60.7	10.
Dextrimaltose (6)	—	—	—	50.7
<i>l</i> -cystine	0.3	0.3	0.3	0.3

(1) Vit-test casein, General Biochemicals.

(2) Hegsted-Mills, Elvehjem and Hart, *J.B.C.*, 1941, v138, 459.

(3) Hydrogenated cottonseed oil, Swift & Co.

(4) Wesson oil.

(5) Made from cornstarch, Corn Products Sales Co.

(6) Mead's Dextri-Maltose #2, Mead Johnson & Co.

\* This work was partially supported by grants-in-aid from E. R. Squibb and Sons, Hoffmann-LaRoche, Inc., and Sharp and Dohme and Co.

1. Hartman, A. M., Dryden, L. P., and Cary, C. A., U.S.D.A. BDIM-Inf-76, July, 1949.

2. Chow, B. F. and Barrows, L. Fed. Proc., (*Am. Inst. Nutr.*), 1950, v9, 354.

3. Bosshardt, D. K., Paul, W. J., and Barnes, R. H., *J. Nutr.*, 1950, v40, 595.

The following vitamin supplements\* were added to all rations:

Dissolved in cottonseed oil and added to 100 g ration

Percormorph oil (Mead Johnson & Co.)	1 drop
<i>a</i> -tocopherol	10 mg
Menadione	1.5 "
Added to 100 g ration	
Thiamine	.3 mg
Riboflavin	.5 "
Pyridoxine	.5 "
Ca. pantothenate	3.0 "
Niacin	5.0 "
Biotin	.015 "
Folic acid	.03 "
Inositol	15.0 "
<i>p</i> -aminobenzoic acid	25.0 "
Choline chloride	125.0 "

\* All of the vitamins used were provided by Merck and Co.

were placed on each ration. In order to obtain animals with reduced stores of B<sub>12</sub> the young rats used were the offspring of adults from our stock colony which had been placed on a B<sub>12</sub>-deficient soybean diet at the time of mating. The females were maintained on this ration during pregnancy and lactation. The young had access to the ration until placed on the experimental diets. The animals were allowed to eat *ad libitum*. Food consumption records were kept. Half of the animals on each ration received 0.5 mcg B<sub>12</sub><sup>†</sup> subcutaneously three times a week. The animals were weighed weekly and were kept in individual metal cages the floors of which were sufficiently elevated to prevent coprophagy.

**Results.** As was expected, the animals receiving the B<sub>12</sub> injections averaged greater weight gains than did their littermates not receiving the vitamin. After 28 days on the diet the greatest average gain in males was made by those on ration A. In the females those on the high carbohydrate diets, C and D, averaged the greatest gains. The differences between the average weight gains made by these animals and those on the same diets without B<sub>12</sub> were considerably greater than those of the males on the same rations. (Table II).

The females on the high carbohydrate diets (C) that were not given B<sub>12</sub>, gained less than those not receiving the vitamin on the

TABLE II. Average Gain in Body Weight.

Diets	Females After 28 days on diets				Males				After 63 days on diets			
	Females		Males		Females		Males		Females		Males	
	B <sub>12</sub>	No B <sub>12</sub>	B <sub>12</sub>	No B <sub>12</sub>	B <sub>12</sub>	No B <sub>12</sub>	B <sub>12</sub>	No B <sub>12</sub>	B <sub>12</sub>	No B <sub>12</sub>	B <sub>12</sub>	No B <sub>12</sub>
Low carbohydrate	86 ± 1.8(5)*	75 ± 5.3(6)	106 ± 8.9(5)	91 ± 11 (3)	143 ± 2.7(7)	143 ± 7.2(5)	218 ± 12.1(3)	205 ± 9.8(5)				
—high fat												
Medium carbohydrate	89 ± 3.8(7)	78 ± 5.4(5)	124 ± 8.7(3)	101 ± 8.1(5)	154 ± 4.2(7)	139 ± 4.0(5)	253 ± 8.1(3)	242 ± 8.1(5)				
—medium fat												
High carbohydrate (dextrin) low fat	87 ± 4.9(7)	56 ± 9.8(5)	112 ± 5.8(3)	95 ± 11.5(4)	155 ± 5.7(7)	108 ± 16.5(5)	246 ± 11.0(3)	212 ± 11.5(4)				
High carbohydrate (dextri-maltose) low fat	96 ± 5.3(6)	70 ± 2.7(6)	103 ± 7.0(4)	83 ± 7.0(4)	170 ± 12.0(6)	139 ± 3.7(6)	228 ± 23.0(4)	206 ± 7.5(4)				

\* Average gain in wt is expressed in g with stand. error of the mean. Number in parentheses indicates number of animals used.

† Rubramin—E. R. Squibb and Sons.

TABLE III. Average Grams of Protein Consumed per Gram Body Weight Gained.

Diets	After 28 days on diets				After 63 days on diets			
	Females		Males		Females		Males	
	B <sub>12</sub>	No B <sub>12</sub>	B <sub>12</sub>	No B <sub>12</sub>	B <sub>12</sub>	No B <sub>12</sub>	B <sub>12</sub>	No B <sub>12</sub>
Low carbohydrate	.94(5)*	.95(6)*	.83(5)	.79(3)	1.40(5)	1.33(6)	1.08(5)	1.10(3)
—high fat								
Medium carbohydrate	1.03(7)	.97(5)	.88(3)	.82(5)	1.50(7)	1.43(5)	1.11(3)	1.12(5)
—medium fat								
High carbohydrate	.99(7)	1.16(5)	.79(3)	.78(4)	1.47(7)	1.49(5)	1.10(3)	1.16(4)
(dextrin)—low fat								
High carbohydrate	.91(6)	1.17(6)	.87(4)	.84(4)	1.33(6)	1.40(6)	1.11(4)	1.09(4)
(dextri maltose)								
—low fat								

\* Figure in parentheses indicates number of animals used.

diets of moderate and high fat content. In the males, on the other hand, this difference was not so marked. The effect of B<sub>12</sub> on weight gain was not as striking among the females on the moderate and high fat diets (A and B) as among those on the two diets high in carbohydrate (C and D). The averages of weight gains made by the females receiving B<sub>12</sub> varied from 86 g on B to 96 g on D. Among males receiving B<sub>12</sub> the gains varied from 103 g on D to 124 g on A. On diet D, high carbohydrate, the weight-stimulating effect of B<sub>12</sub> is least marked in males and most marked in females whereas on ration A, moderate carbohydrate and moderate fat, the reverse is true. Among females not receiving B<sub>12</sub> the differences in weight gains between those on the high carbohydrate diets and those on the moderate and high fat diets were somewhat greater than the differences between the average gains of those females receiving B<sub>12</sub> and those not receiving the vitamin on the moderate and high fat diets. From this it appears that in young, growing, female rats, fed diets high in carbohydrate, B<sub>12</sub> exerts a greater effect on weight gain than it does on littermate males on the same diets. This suggests that B<sub>12</sub> is involved in the conversion of carbohydrate into fat. Since females in general have a higher percentage of fat in proportion to total body weight than do males it might be expected that greater weight gains would result in them than in males upon the administration of a factor concerned with the conversion of carbohydrate into fat.

After 28 days on the diet, except among the females on the high carbohydrate diets, (C and D), there were no significant differences in grams of protein consumed per gram of weight gain between the animals receiving B<sub>12</sub> and those not receiving it. In every case on all the rations, both with and without B<sub>12</sub>, the average amount of protein required per gram of weight gain was greater for females than for males. (Table III).

The data obtained after 63 days serve to extend those collected after the animals had been on the diets for 28 days. The trends are approximately the same at the end of the longer period as those noted for the first 28 days. (Table II). The most apparent difference between the results at the ends of the 2 periods is in the amount of protein required per gram of weight gain. After 63 days in both males and females, on all 4 rations, with and without B<sub>12</sub>, the amount of protein required per gram of weight gain is markedly increased over that required during the first 28 days on the diets.

**Summary.** Comparison of the average weight gains made by young, growing rats (offspring of B<sub>12</sub>-depleted animals) on diets of varying fat and carbohydrate content, with and without B<sub>12</sub>, shows that subcutaneous administration of B<sub>12</sub> produces increased weight gains in both sexes on all four of the rations studied. It appears that when the calories are derived primarily from carbohydrate the vitamin exerts a greater effect on weight gain in young, growing, female rats than in their littermate males. The weight-

stimulating effect of  $B_{12}$  is more marked in females on high carbohydrate diets than in littermate sisters on diets of like protein con-

tent but with the calories provided by fat or a mixture of fat and carbohydrate.

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### Suppression of Vasomotor Reflexes in Man Following L-Hydrazinophthalazine (C-5968).\* (18085)

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A recent paper by Reubi(1) indicates that L-hydrazinophthalazine (C-5968), produces an increase in renal blood flow in hypertensive patients as well as a variable reduction in arterial pressure. Since no other hypotensive agent thus far studied possesses such properties(2) it seems of interest to attempt to localize further the site of action of C-5968 in man.

*Method and results.* L-hydrazinophthalazine was administered intravenously to 12 normotensive and 3 hypertensive patients in doses of 0.30 to 0.35 mg per kg. Sympathetic vasopressor responses, digital plethysmography, and skin temperature recordings were carried out according to methods previously described(3). Following intravenous injection of the phthalazine derivative all of the changes described below began to develop after 10 minutes, reached a maximum in 20 to 40 minutes, and then slowly receded over a period of several hours. The reduction in

systolic + diastolic

mean ( $\frac{1}{2}$ ) arterial pres-  
2

sure ranged from 0 to 24% (average 8.5%) in the normotensive subjects and from 0 to 23% (average 8.6%) in the hypertensive patients. In both groups the percentile reduction of diastolic pressure was greater than the percentile fall in systolic pressure. In all patients the visible precordial and carotid pulsations appeared more prominent and forceful. A moderate tachycardia appeared in all patients (pulse rate increase 4 to 32 beats per minute) except 2 of the hypertensive patients who developed bradycardia. Transient inversion of the T waves particularly in leads V<sub>2</sub> to V<sub>5</sub> frequently were observed in the electrocardiogram.

After C-5968 there usually occurred marked inhibition or abolition of the vasopressor "overshoot" following the Valsalva maneuver, the tiltback "overshoot" and the cold pressor response. Postural hypotension occurred frequently but not invariably. Significant changes in digital skin temperature were not observed in a room maintained at 70°F. However, in environmental temperatures of 75° and higher, flushing of the hands frequently was seen, and there was an increase in pulse volume of both fingers and toes. The reflex vasoconstrictor responses to "noxious" stimuli in the digits were significantly inhibited.

Preparation C-5968 in the dosages used in the present studies in man did not prevent or reverse the hypertension produced by commercial epinephrine infused intravenously at rates of 0.11 to 0.23  $\mu$ g per kg per min. Also, the tachycardia produced by epinephrine was not prevented. However, in 3 of 3 patients studied when norepinephrine was

\* Supported in part by research grants from the National Heart Institute, U. S. Public Health Service, The Squibb Institute for Medical Research, New Brunswick, and Irwin Neisler and Company, Decatur. Preparation C-5968 supplied by CIBA Pharmaceutical Products.

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given intravenously at rates varying between 0.15 and 0.30  $\mu\text{g}$  per kg per min. there was a moderate reduction of the pressor response varying between 12 to 45% (mean 31%). In addition there was a consistent and marked (approximately 80%) inhibition of the bradycardia induced by norepinephrine. In one instance, following C-5968, the infusion rate of norepinephrine was raised to twice that given in the control period. This resulted in a 31% increase in mean arterial pressure without, however, significant change in heart rate. Thus, after C-5968, blockade of norepinephrine bradycardia appeared to be far more complete than the inhibition of the pressor response induced by norepinephrine.

The side effects were palpitation, frequently observed, headaches, complained of in three subjects and dyspnea, which was noted by 2 hypertensive patients. The headaches were of a severe "pounding" type usually located in the occipital area, and were observed more frequently in patients receiving prolonged oral medication than in the present series given single intravenous injections of the drug.

**Discussion.** The significant although incomplete inhibition of sympathetic reflexes following C-5968 indicates that the drug has autonomic blocking action. The site of inhibition may be central, ganglionic or peripheral. The absence of epinephrine reversal and nasal congestion suggest that this compound in the dosages used does not act at the peripheral nerve endings. These observations in man are opposite to those achieved with higher dosage levels in animals by Gross and his co-workers(4). Others(5,6) have observed diminution in the epinephrine pressor response in animals but only in doses higher than those used in the present studies in man. There have been no reports of epinephrine reversal. Thus, preparation C-5968 does not appear to be adrenolytic in the

same sense as Dibenamine or Priscoline. However, the slight inhibition of the norepinephrine pressor response observed in the present study as well as the inhibition of both epinephrine and norepinephrine hypertension following larger doses of C-5968 in animals(4-6) suggest that the drug may have an unusual type of peripheral action.

The failure to produce potentiation of the epinephrine and norepinephrine pressor responses, and the absence of changes either in pupillary size or visual accommodation suggests that the drug does not block ganglionic transmission. The other possible sites of action are in the central nervous system including the hypothalamus, the medulla and/or the spinal cord. Central inhibition of vasomotor reflexes is suggested by the ability of C-5968 to block almost completely the bradycardia following norepinephrine since the latter response probably is due to activation of the carotid sinus and aortic arch reflexes secondary to the hypertension produced by norepinephrine. Compound C-5968 differs, however, from other drugs which inhibit vasomotor reflexes centrally such as dihydroergocornine(6) and pentaquine(7) in that it also produces palpitation and tachycardia to a degree almost as marked as that observed after epinephrine.

Like DHO, preparation C-5968 did not act immediately after intravenous injection but required a latent period of 10 to 15 minutes. This suggests that the drug either is converted to an active compound in the body, or that the central mechanism which it affects responds slowly or else is acted upon secondarily through a chain reaction affecting unknown intermediary organs.

**Summary and conclusions.** The administration of L-hydrazinophthalazine results in inhibition of sympathetic vasoconstrictor reflexes in man. In addition it has epinephrine-like effects on the heart. C-5968 differs from other "sympatholytic" drugs studied thus far in man.

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**Intracavitary Visceral Radiation: Effect on Gastric Acid Secretion.  
(18086)**

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A transient depression of gastric function has been noted following administration of large doses of radioactive iodine for thyroid carcinoma(1). This observation suggested the possibility that a reduction in gastric acid secretion might be achieved by radiation of the parietal cells from a radioactive isotope placed within the stomach cavity in such a manner as to preclude absorption.

Since 1917 various workers [listed by Palmer(2)] have used roentgen rays to reduce gastric acidity in treatment of peptic ulcer. Palmer's group have reported favorably from their extensive experience with its use(3-6). The chief disadvantage of roentgen radiation is the considerable radiation received by tis-

sues other than the target tissue during the course of treatment.

In attempting to apply radiation from radioisotopes to the gastric mucosa the use of a balloon powdered on the inside surface with radioactive dust was considered but technical difficulties in the safe preparation and manipulation of such a device led to the development of a modification permitting use of the radioactive isotope in solution. It was first planned to distend the bag within the stomach by introduction of radioactive solution but the dangers in handling the large volume of solution required, particularly since beta rays only reach the mucosa from the 2-3 mm of solution closest to the stomach

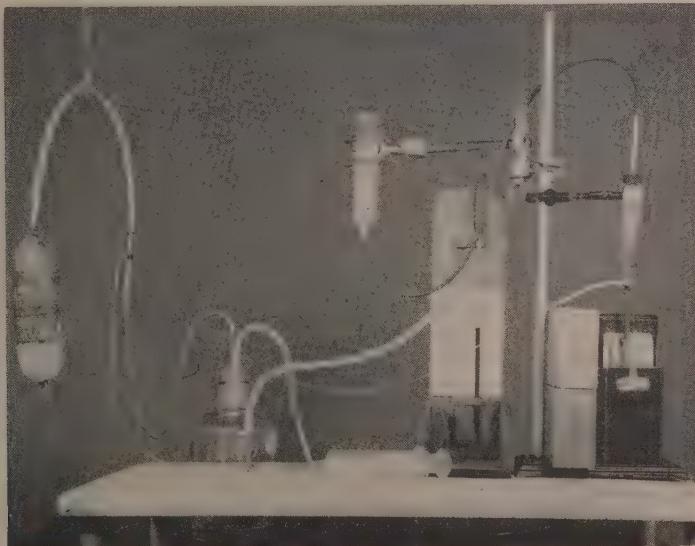


FIG. 1.  
The assembled apparatus.

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## INTRACAVITY VISCERAL RADIATION ON GASTRIC ACID

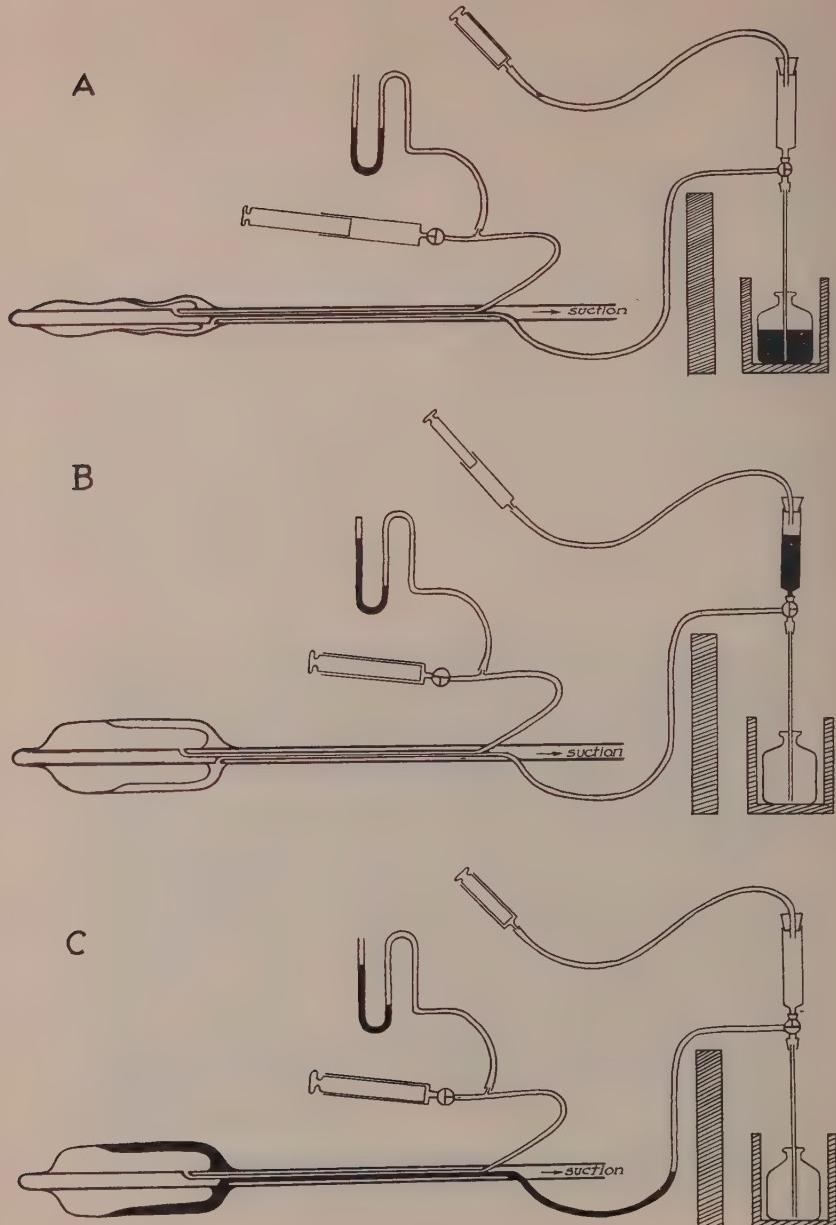


FIG. 2, A, B, C. Procedure for inflation of intra-gastric bag, and introduction of radioactive isotope solution into the intermural compartment.

wall, led to further modification of the apparatus. A double-walled bag was constructed with an intermural compartment of small

capacity (10-12 cc). The latter feature was achieved by fastening the two layers of rubber together by dots of cement so arranged

that there was about one dot per square centimeter on the distended bag. Thus, the proximal 2/3 of the wall of the bag (that part in contact with the acid-bearing segment of stomach) forms a miniature air mattress into which radioactive solution can be introduced through a fine plastic catheter after the bag has been placed and inflated with air.

It would be expected that almost any isotope emitting predominantly high-energy beta rays would be suitable for use. However, radioactive iodine has particular advantages: (1) it is probably the most extensively used and widely available isotope in clinical practice; (2)  $I^{131}$  emits beta rays of fairly high energy; (3) a unique property is that the uptake of  $I^{131}$  by the body can be largely blocked by pre-treatment of the subject with an anti-thyroid drug. The latter is of practical importance in that spillage of radioactive material from accidental rupture of the bag might be catastrophic if the isotope was freely absorbed and persistently retained. As a further safeguard against such an eventuality, the apparatus has been so arranged as to permit constant suction through the tip of the gastric tube distal to the bag.

The apparatus in its present form is depicted in Fig. 1 and 2. Specially prepared adapters (Fig. 3) fastened into a gastric aspiration tube (Davo 728) permit the entry and exit of 2 fine plastic catheters. One catheter communicates with the interior compartment of the bag, permitting it to be inflated after introduction into the stomach. The function of the manometer on this line is to signal the possible occurrence of rupture of the bag. The other catheter communicates with the intermural compartment and permits introduction and withdrawal of radioactive solution by remote manipulation of a syringe.

*Applications of the technic.* To date, use of the apparatus has been limited to irradiation of the stomach of experimental animals. Achlorhydria has been successfully induced in several dogs without evident damage to the general health of the animals. Experiments are continuing to determine the required dosage of radiation, the duration of



FIG. 3.

Detail of metal adapters and plastic connecting tubes placed within the lumen of the gastric tube.

the achlorhydria induced and to correlate the histologic appearances of serial gastric biopsies with the changes in hydrochloric acid secretion.

*Summary.* An apparatus has been described permitting intracavitory radiation of hollow viscera accessible to intubation. In preliminary trials the technic has been used to induce achlorhydria in the stomach of dogs. This technic may have additional practical applications for lesions in the rectum, bladder, bronchi and other organs or tracts accessible to intubation.

The valuable suggestions of Dr. A. C. Corcoran and Dr. Otto Glasser, and the technical assistance of Dr. C. Ballinger are gratefully acknowledged. Assistance in fabrication of the intragastric bag was provided by the American Anode, Inc., Akron, Ohio.

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## Plasma Levels of Free Amino Acids in Normal Subjects Compared with Patients with Rheumatoid Arthritis.\* (18087)

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Evidence has been presented recently to show that abnormal metabolism of amino acids may occur in rheumatoid arthritis. DeVries and Alexander(1) have reported low plasma glycine in some cases of rheumatoid arthritis. We have reported increased plasma levels as well as increased urinary excretion of histidine in patients with rheumatoid arthritis treated with adrenocorticotropic hormone and with 17-hydroxy-11-dehydrocorticosterone(2). Urinary excretions of other amino acids in patients with this disease have also been reported(3-5).

The present investigation was undertaken to determine whether a difference exists between plasma levels of "free" amino acids of normal individuals and patients with rheumatoid arthritis. The plasma levels of "free" arginine, glycine, histidine, lysine, phenylalanine, serine and threonine will be reported.

**Experimental.** The control subjects were research associates who were apparently normal and in good health. The patients with rheumatoid arthritis had been under observa-

tion of this group from 6 months to 15 years. The average age of the control males was 34.2 years in contrast to 40.6 years for males with rheumatoid arthritis. The average age for the control females was 34.7 years compared to 50.3 years for females with rheumatoid arthritis—a difference of 15.6 years. Ackermann, Hoffstatter, and Kountz(6) have reported that no difference exists in the plasma histidine values in groups with as great a variation in age as 46.7 years. With few exceptions patients with rheumatoid arthritis were hospitalized for observation and treatment at the time this study was begun. All forms of medication were discontinued for 3 days prior to the time the blood samples were taken. The patients were on a rigidly controlled diet containing 70 g of protein per day. The diet of the control group was adequate but no attempt was made to keep an accurate account of protein intake. Kirsner, Sheffner and Palmer(7) have reported that a variation of as much as 35 g of protein per day had no significant effect on the plasma levels of the "free" amino acids we will report in this paper. After a 12-14 hour fasting period, 30 ml of blood were obtained by venipuncture and prevented from clotting by the use of heparin. Plasma tungstic acid filtrates were prepared according to the method of Hier and Bergeim(8). The filtrates were stored at -15°C. At the time of assay they were thawed, adjusted to a pH of 6.9 and diluted so that the final volume represented a 1:5 or 1:10 dilution of the original

\* This work was supported in part by grants from the United States Public Health Service, and the Fair Foundation.

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TABLE I. Plasma Levels of 7 "Free" Amino Acids.

Amino acid	Normals		Rheumatoid arthritics		
	No. of cases	Mean $\pm$ S.E.	No. of cases	Mean $\pm$ S.E.	Value of "t"
Men					
Arginine	8	22.1 $\pm$ 1.58 (16.4-29.3)	21	15.6 $\pm$ 1.23 (6.7-24.3)	3.25*
Glycine	8	26.0 $\pm$ 2.05 (15.7-34.0)	9	24.0 $\pm$ 2.71 (17.1-40.4)	0.59
Histidine	12	15.2 $\pm$ 0.54 (12.2-18.2)	23	11.1 $\pm$ 0.51 (6.8-16.9)	5.50*
Lysine	8	32.3 $\pm$ 1.63 (25.9-39.2)	21	29.5 $\pm$ 1.60 (14.6-41.6)	1.23
Phenylalanine	10	12.9 $\pm$ 1.43 (7.5-23.9)	17	12.6 $\pm$ 1.00 (6.1-23.8)	0.17
Serine	6	11.4 $\pm$ 2.07 (7.4-20.3)	13	11.8 $\pm$ 1.23 (5.4-20.5)	0.17
Threonine	8	20.7 $\pm$ 2.61 (13.2-36.9)	21	13.2 $\pm$ 0.80 (8.8-19.5)	2.75*
Women					
Arginine	15	22.1 $\pm$ 0.95 (17.2-29.1)	28	14.8 $\pm$ 1.04 (5.3-22.8)	5.19*
Glycine	22	27.1 $\pm$ 1.71 (14.3-49.5)	16	28.5 $\pm$ 3.00 (14.8-60.2)	0.41
Histidine	28	13.9 $\pm$ 0.39 (9.5-17.8)	38	10.8 $\pm$ 0.54 (3.2-18.1)	4.61*
Lysine	18	28.5 $\pm$ 1.68 (17.1-38.6)	26	26.3 $\pm$ 1.57 (12.2-38.5)	0.96
Phenylalanine	21	10.9 $\pm$ 0.46 (8.1-15.6)	29	10.9 $\pm$ 0.39 (6.9-15.1)	0.00
Serine	15	13.3 $\pm$ 0.95 (6.0-18.1)	21	12.9 $\pm$ 1.04 (4.6-23.4)	0.29
Threonine	18	17.9 $\pm$ 1.21 (7.9-27.4)	28	13.0 $\pm$ 0.40 (9.4-17.9)	3.58*

\* Highly significant P less than 0.01.

plasma. It was found that by diluting more than the original 1:3 volume as recommended by Hier and Bergeim(8) the microbiological response fell on the steeper and more sensitive part of our standard curve. Furthermore, the higher dilutions enabled us to run more assays on each individual. The assay procedure and media, with slight modifications, were those of Henderson and Snell(9). Glycine, histidine, lysine, phenylalanine and serine were measured with *Leuconostoc mesenteroides* P-60; threonine, and arginine with *Streptococcus fecalis* R. The filtrates were assayed in duplicate and at three levels. In all other details the procedures as previously described(8,9) were followed. As a check on the precision of the method in our laboratory, assays in quadruplicate were made on pooled tungstic acid filtrates and yielded us

the same " $\pm$  10% of the mean" as reported by Hier and Bergeim(10). In order to determine the effect of freezing and thawing on plasma filtrates duplicate assays were made on 5 pooled filtrate aliquots. One of the aliquots was stored without freezing in a refrigerator at 10°C while the other 4 were frozen and thawed from one to 4 times. The differences from the means fell well within the ( $\pm$ ) 10% accuracy of the method.

**Results.** Table I compares the amount of 7 "free" amino acids found in the plasma of normal individuals with values found for patients with rheumatoid arthritis. It will be seen that no significant differences exist between the values for males and females in either group. The amounts of arginine, histidine, and threonine are significantly lower in the group with rheumatoid arthritis. The

values for glycine, lysine, phenylalanine and serine do not show a significant difference.

*Discussion.* It is generally conceded that plasma values for various amino acids are relatively constant(7). It seems, therefore, that any significant differences are worthy of consideration. Although the changes we have demonstrated may be small in gamma per ml, they are nevertheless valid as shown by statistical analysis. The normal values established in this study compare favorably with those found in the literature(6,7,10).

*Summary.* Microbiological determinations of plasma values for arginine, glycine, histidine, lysine, phenylalanine, serine, and threonine have been determined in a group of nor-

mal individuals and compared with the values obtained from patients with rheumatoid arthritis.

1. The values obtained for arginine, histidine, and threonine in patients with rheumatoid arthritis were lower than those obtained from the normal group. These differences were highly significant(11). 2. The values for glycine, lysine, phenylalanine and serine were not significantly different in the group with rheumatoid arthritis from those found in the group of normal individuals.

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## Free Amino Acids in Sea-Urchin Eggs and Embryos.\* (18088)

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Free amino nitrogen has been determined in embryos of various animals; however, as yet there has been little attempt to analyze the sources of this qualitatively. Li and Roberts(1), using the paper chromatographic method, found no free amino acids in eggs and embryos of the frog. Drilhon and Busnel (2), also using chromatographic methods, found free glutamic acid, serine, alanine, and valine in the eggs of *Bombyx mori*. In later stages of development other amino acids appeared.

The present work was undertaken to investigate the occurrence of free amino acids in sea-urchin eggs, and to determine whether qualitative changes take place during development. Unfertilized sea-urchin (*Strongylocentrotus purpuratus*) eggs were extracted with 75% ethanol at 3-5°C for 2 days; 1.5

cc of ethanol were used per 50 mm<sup>3</sup> of eggs packed by centrifugation. The supernatant was analyzed for amino acids and peptides by means of 2-dimensional paper chromatography as described by Consden *et al.*(3). Whatman filter paper No. 1 was used with water saturated phenol as the first solvent and a n-butanol-acetic acid-water mixture (approximately 11:3:4) as the second. Amino acids and peptides were revealed by spraying the papers with 0.2% ninhydrin and heating to approximately 80°C for 10-20 minutes. Amino acid spots were tentatively identified by position and checked by adding pure amino acids to the unknown.

Free amino acids identified in the unfertilized sea-urchin eggs were glycine, glutamic acid, lysine, threonine, alanine, valine, leucine (or isoleucine). A ninhydrin-positive spot with R<sub>f</sub> values slightly less than alanine was found to be glutamine. Addition of pure glutamine to the extract caused intensification of this spot. Acid hydrolysis of the material

\* This work was supported in part by the Division of Medical Physics and the Radiation Laboratory under the auspices of the United States Atomic Energy Commission.

1. Li, C., and Roberts, E., *Science*, 1949, v110, 425.

2. Drilhon, A., and Busnel, R., *Comp. Rend. Acad. Sci., Paris*, 1950, v230, 1114.

3. Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, 1944, v38, 224.

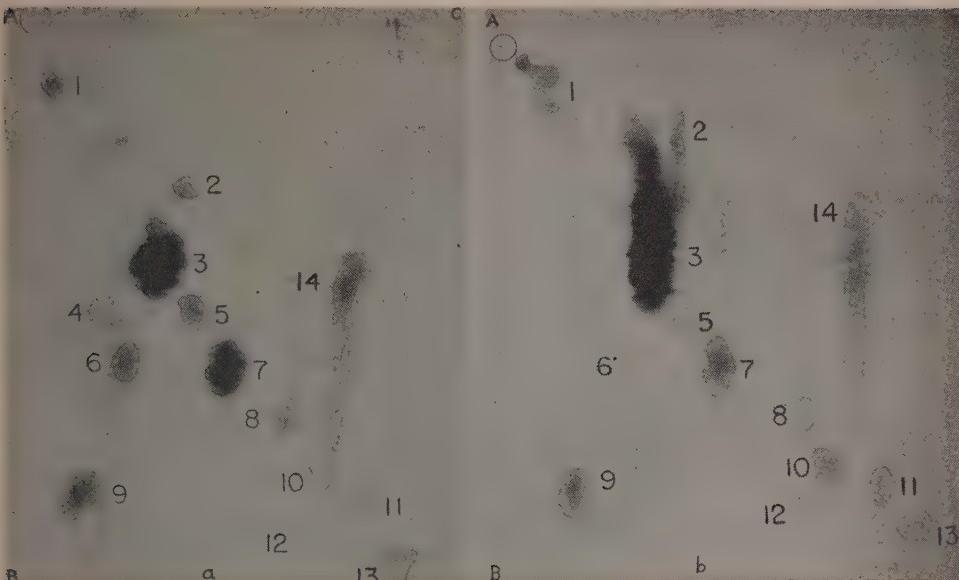


FIG. 1.

Paper partition chromatograms of extracts of sea-urchin eggs and embryos. The ethanol extracts of 25,000 eggs or embryos were placed in the circles in the upper left hand corners. *a*, unfertilized eggs; *b*, prism larvae. A-B, water saturated phenol; A-C, n-butanol-acetic acid-water. Spots (circled with pencil): 1, peptides; 2, glutamic acid; 3, glycine; 4, lysine; 5, threonine; 6, glutamine; 7, alanine; 8, peptide; 10, valine; 11, leucine (or isoleucine); 13, peptide; 9, 12, 14, unhydrolyzable ninhydrin-positive substances.

eluted from this area resulted in the disappearance of glutamine and the appearance of glutamic acid.

Several peptides were found with widely different  $R_f$  values. These disappeared after acid hydrolysis (refluxed with 6 N HCl for 20 hours) of the extracts and new amino acids appeared and the color of pre-existing marks was intensified. The probable peptide nature of these spots was further investigated by eluting them from a one dimensional phenol chromatogram. The eluate was hydrolyzed with acid and the hydrolyzate chromatographed to check the presence of amino acids.

A typical 2-dimensional chromatogram of the free amino acids and peptides obtained from unfertilized sea-urchin eggs is shown in Fig. 1a. Glycine is present in a relatively large amount as indicated by the size and dark color of its spot. Alanine is less concentrated and the remaining amino acids appear as faint spots indicating a comparatively

low concentration.

Since spot No. 1 often separates into two areas, it may represent more than one peptide. The amino acids in hydrolyzates of the material eluted from this area have been tentatively identified as glutamic acid, cysteine or cystine, glycine or serine, and possibly aspartic acid. Pure glutathione when added to an extract did not coincide with this spot.

Addition of  $\alpha$ -amino butyric acid caused intensification of spot No. 8, which, however, did not appear in acid hydrolyzates of the extracted spot. Instead, a number of amino acids were obtained. The disappearance of spot No. 13 after acid hydrolysis indicates that it is also a peptide. Glycine, serine, alanine and possibly other amino acids are obtained after elution and hydrolysis of this peptide.

Spot No. 14 is an unidentified substance which initially appears as a yellow streak after spraying with ninhydrin, but which becomes dark purple within a few hours. It

is not affected by acid hydrolysis. Spot No. 9, also unidentified, often partially separates into 2 areas and may consist of several substances with similar  $R_f$  values.

Ethanol extractions of amino acids and peptides from various stages of development (unfertilized eggs, early cleavage, swimming blastulae, gastrulae, and prism larvae) with comparable amounts of material (250,000 eggs or embryos per cc of extract) indicate that there is no striking qualitative change during development.

A typical chromatogram of extracts of a late stage of development is shown in Fig. 1b. No new amino acids appear on the chromatograms of these later embryonic stages and a few (alanine, lysine and glutamine) apparently decrease in concentration. In chromatograms of gastrulae and prism larvae glycine and glutamic acid often spread out more in the direction of the phenol run (Fig. 1b) which suggests an increase in concentration of these amino acids. Aside from these

slight changes the patterns of amino acids and peptides on chromatograms of later embryonic stages are similar to those of unfertilized eggs.

*Summary.* The free amino acids of sea-urchin (*Strongylocentrotus purpuratus*) eggs and embryos were extracted with 75% ethanol and identified by means of paper chromatography. Extracts of unfertilized eggs contain relatively large amounts of glycine and small amounts of alanine, glutamic acid, valine, threonine, lysine, glutamine, and leucine (or isoleucine) as well as several peptides and unknown ninhydrin-positive substances. The patterns of amino acid and peptide spots on chromatograms of extracts of cleavage stages, blastulae, gastrulae and prism larvae are dissimilar to that of unfertilized eggs.

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## Studies in Protein Metabolism of the Amphibian Embryo. II. Free Amino Acids. (18089)

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The method of paper chromatography(1) has only recently been used for the identification of free amino acids in eggs and embryos, namely, by Drilhon and Busnel(2) for *Bombyx mori*, Agrell(3) for *Calliphora erythrocephala*, and Berg(4) for *Strongylocentrotus purpuratus*. Li and Roberts(5), however, were unable to demonstrate any free amino acids in the developmental stages of the frog [presumably *Rana pipiens*] ranging from

the unfertilized egg to the young non-feeding tadpole. They detected peptides, however, which upon hydrolysis yielded small quantities of aspartic acid, glutamic acid, glycine, and alanine.

We have examined two amphibians, *Rana pipiens* and *Hyla regilla*, for free amino acids in 4 stages of development: zygote (or, in case of *Hyla*, early cleavages), beginning gastrula, middle neurula, and young tailbud embryo. In terms of numbered stages described by Shumway(6) for *Rana pipiens* and by Eakin(7) for *Hyla regilla* the stages used were 2 (or, in case of *Hyla*, 2 to 7), 10, 14,

1. Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, 1944, v38, 224.
2. Drilhon, A., and Busnel, R., *Comp. Rend. Acad. Sci., Paris*, 1950, v230, 1114.
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4. Berg, W. E., *PROC. SOC. EXP. BIOL. AND MED.*, 1950, v75, 30.
5. Li, C., and Roberts, E., *Science*, 1949, v110, 425.

6. Shumway, W., *Anat. Rec.*, 1940, v78, 139.

7. Eakin, R. M., *Univ. Calif. Publ. Zool.*, 1947, v51, 245.

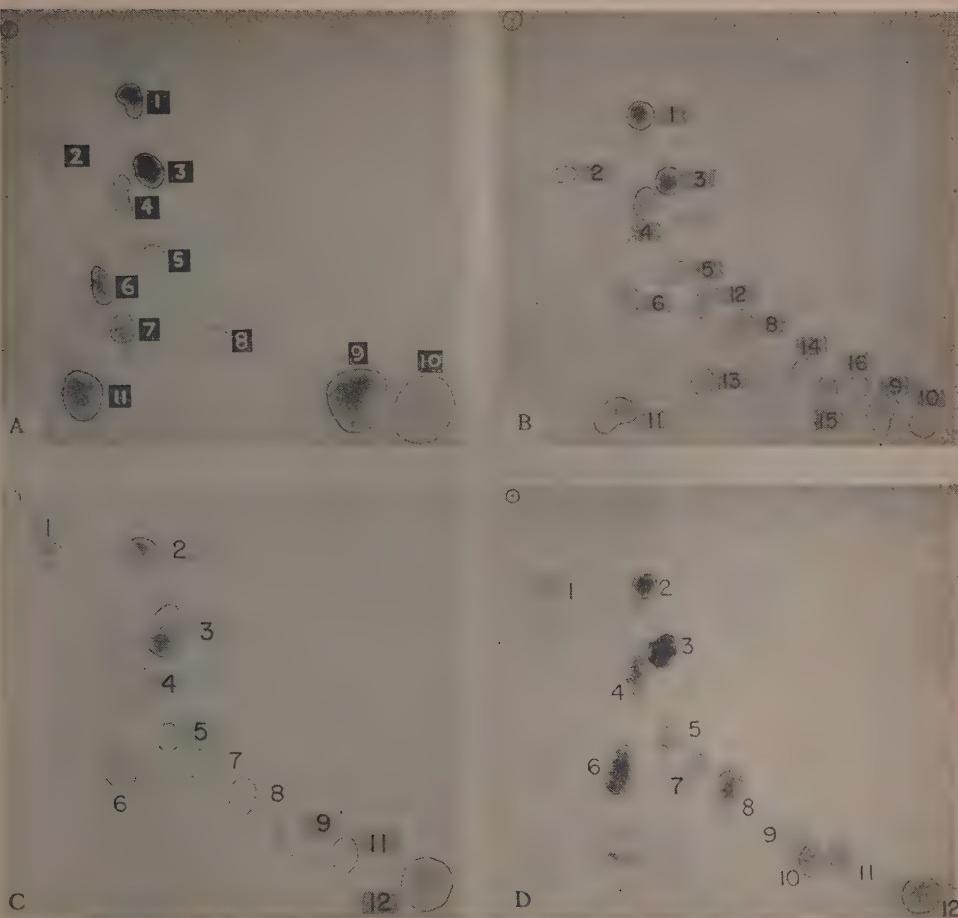


FIG. 1. Chromatograms of Ethanol Extracts.

A, *Rana pipiens* zygotes (stage 2); B, *R. pipiens* tailbud embryos (stage 18). Spots (circled with pencil): 3, glutamic acid; 4, serine; 5, threonine; 6, glutamine; 7, methionine sulfoxide (?); 8, tyrosine; 12, alanine; 14, valine; 15, phenylalanine; 16, leucine (or isoleucine); 1, 9, and 10, peptides; 2, 11, and 13, unidentified.

C, *Hyla regilla* early embryos (stages 2-7); D, *H. regilla* tailbud embryos (stage 18). Spots (circled with pencil): 3, glutamic acid; 4, serine; 5, threonine; 6, glutamine; 7, alanine; 8, tyrosine; 9, valine; 10, phenylalanine; 11, leucine (or isoleucine); 2 and 12, peptides; 1, unidentified.

Movement of solvents: phenol-water, vertical direction; n-butanol-acetic acid-water, horizontal direction. Spots in Fig. C not well shown because photograph was made with direct light; photographs A, B, and D taken with transmitted light. Photographs made by Victor G. Duran.

and 18. The embryos were first rendered free of most of the jelly; tailbud embryos were removed from the chorion. For each stage of both species 500-1300 embryos were homogenized in a final concentration of 75% ethyl alcohol. Extraction by the alcohol was continued at 2°C for 48 hours. Samples were

centrifuged. The supernatant from each was decanted off, evaporated to dryness, and brought by the addition of distilled water to a final concentration of 167 (*Rana*) or 200 (*Hyla*) embryos per ml. The paper chromatographic method(1,4) was employed. The solvents used were phenol saturated with

water and a mixture of n-butyl alcohol, acetic acid, and water (approximately 11:3:4).

The results are illustrated in Fig. 1. Spots 1, 9 and 10 in *Rana* (Fig. 1A-B) and marks 2 and 12 in *Hyla* (Fig. 1C-D) are assumed to be peptides since they disappeared upon acid hydrolysis (refluxed with 6N HCl for 20 hours). They will be analyzed in a later study.

*Rana* and *Hyla* chromatograms are remarkably similar. *Rana*, but not *Hyla*, shows a spot tentatively designated methionine sulfoxide (Fig. 1A, No. 7). Methionine added to the extract of *Rana* zygotes intensifies this spot, presumably by the oxidized form and a new spot (unoxidized methionine) appears to the right and in the position of valine. Valine and methionine have similar R<sub>f</sub> values, but the mark designated valine on chromatograms of older material probably is not methionine. The methionine sulfoxide spot is absent or very faint in tailbud papers whereas the valine spot has by that stage become very definite. Alanine, leucine (or isoleucine), and valine present in *Rana* after the beginning of gastrulation, can be detected in the youngest *Hyla* material. Unfortunately because of the difficulty of procuring an adequate quantity of *Hyla* zygotes, early cleavage stages were also included so that the early *Rana* and *Hyla* extracts are not strictly comparable.

There is no marked change in the picture as development proceeds. In both species the large peptide spots decrease in intensity;

phenylalanine first appears in neurulae in *Rana* and in gastrulae in *Hyla*; and in *Rana* leucine (or isoleucine) and valine are first detectable in gastrular extracts. In addition, the spot on *Rana* papers tentatively identified as oxidized methionine is so faint on stage 18 chromatograms that it was not circled in the photograph (Fig. 1B).

Finally we report one trial experiment in which extracts of dorsal lip explants in *Hyla* were compared chromatographically with those of explants of ventral ectoderm. For this one run approximately 300 explants of each type were needed. The explants corresponded to those used by Friedberg and Eakin(8) in their study of glycine uptake. The two chromatograms were essentially alike in the number and intensity of the spots and agreed with the picture for the whole gastrula.

**Summary.** Several free amino acids and one amide were identified by paper chromatography in alcoholic extracts of eggs and early developmental stages of *Rana pipiens* and *Hyla regilla*. Other ninhydrin-positive substances, including several peptides, will be studied later. One experiment on *Hyla* revealed no major chromatographic difference between explants of dorsal lip and ventral ectoderm.

8. Friedberg, F., and Eakin, R. M., *J. Exp. Zool.*, 1949, v110, 33.

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## The Effect of Cyclopropane, Ether, and Thiopental Sodium upon the Over-Digitalized Heart.\* (18090)

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Since cyclopropane tends to shift upward

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the pacemaker which has been displaced by digitalis preparations(1), it seemed of interest to investigate the effect of certain other anesthetics. This report deals with a comparison of the effects of cyclopropane, ether

1. Stutzman, J. W., Allen, C. R., and Meek, W. J., *Anesthesiology*, 1942, v3, 644.

and thiopental sodium<sup>†</sup> upon the cardiac arrhythmias caused by over-digitalization.

**Procedure.** Twelve adult mongrel dogs, weighing between 5 and 13 kg, were used in this study. Four dogs were digitalized by the intravenous injection of digitoxin,<sup>†</sup> 0.3 mg/kg. Eight dogs were digitalized by the intravenous injection of Strophanthin G (Arnaud's Ouabaine),<sup>‡</sup> 0.07 or 0.08 mg/kg each day that they were used. Criteria for over-digitalization were (a) the production of arrhythmias, (b) the prolongation of the PR interval, or (c) alterations of the ST segment from the position seen in the control electrocardiogram. All dogs vomited following the administration of the digitalis preparations, and in each dog at least one of the signs of cardiac toxicity was seen. It was found that the abnormalities induced by digitoxin persisted for days. The maximum effect of ouabaine was seen in one hour and arrhythmias persisted for 5 to 7 hours. Dogs were not digitalized with ouabaine more often than once in 7 days.

An electrocardiogram, Standard Lead II, was taken before and after digitalization, during anesthesia after 30 minutes equilibration, and upon recovery from anesthesia. Recovery was deemed to be the time the animal could stand and walk, and was at least 30 minutes after the administration of the anesthetic was discontinued.

Each anesthetic was given to 10 dogs. Eight of the dogs received all 3 anesthetics. Cyclopropane and thiopental sodium were administered the same day to each dog, with an interval of at least 2 hours between recovery from the first and induction with the second anesthetic. The order of administration of the two agents was varied. Ether was never given within 24 hours of any other anesthetic and was always the last agent tested.

Dogs were anesthetized with cyclopropane by rebreathing a cyclopropane-oxygen mixture

via mask from a 5 liter bag. An endotracheal tube with an inflatable cuff was then inserted to insure a closed system and an open airway. The system was connected through a soda-lime carbon dioxide absorber to a large rubberized bag containing a 28 to 31 per cent mixture of cyclopropane in oxygen. Ether anesthesia was induced by drop on a gauze mask. An endotracheal tube with an inflatable cuff was inserted and connected with a Wolff bottle adjusted for maintenance. Thiopental sodium, 30 mg/cc, was injected intravenously in average total dosage of 25 to 30 mg/kg. An effort was made with each agent to maintain a comparable depth of surgical anesthesia with partial intercostal paralysis. Animals were maintained at this level for 30 minutes and then allowed to recover.

**Results and discussion.** In Table I are summarized the effects of each of the 3 anesthetics on the over-digitalized heart. Under cyclopropane anesthesia 8 of the 10 dogs showed improvement in the digitalis-induced arrhythmias. Upward displacement of the pacemaker to the SA node was seen in 4 dogs; upward displacement of the pacemaker to the AV node from ventricular foci occurred in 1 dog; and reduction to control levels of the PR interval, with consequent disappearance of the partial AV block, was shown in 3 dogs. Two had no improvement. All dogs demonstrated a return to or maintenance of pre-anesthetic abnormalities upon recovery from anesthesia.

Digitalis has been reported to produce cardiac arrhythmias by two mechanisms. In smaller dosage, reflex vagal stimulation(2) and direct depression of the conduction time account for the observed irregularities(3). In higher dosage the effect upon the myocardium becomes predominant, causing an increase in irritability which is manifested by the appearance of ectopic beats and rhythms (4).

2. Heymans, C., Bouckaert, J. J. and Regniers, P., *Compt. rend. soc. de biol.*, 1932, v110, 572.

3. Gold, H., Kwit, N. T., Otto, H., and Fox, T., *J. Pharm. and Exp. Therap.*, 1939, v67, 224.

4. Cushny, A. R., *Digitalis and Its Allies*, London, Longmans, Green and Co., 1925.

<sup>†</sup> Thiopental sodium and Digitoxin used in this study generously supplied by Abbott Laboratories, North Chicago, Ill.

<sup>‡</sup> Arnaud's Ouabaine generously supplied by E. Fougera Company, New York City.

TABLE I. Effect of Cyclopropane, Ether, and Thiopental Sodium on the Electrocardiogram of the Over-digitalized Animal.

	Cyclopropane series		Ether series		Thiopental sod. series	
	No. of animals	Rate avg (range)	No. of animals	Rate avg (range)	No. of animals	Rate avg (range)
Control						
SA	10	116 (98-130)	10	122 (100-158)	10	116 (98-130)
Digitalized						
SA	2	135 (130-140)	2	108 (90-125)	2	125 (110-140)
SA c partial AV block	2	123 (112-135)	5	92 (66-114)	5	93 (73-110)
SA c V Exs	1	65	0		0	
AV	0		0		1	180
Shifting pacemaker	2	146 (132-160)	1	93	0	
V	3	225 (210-240)	2	202 (184-220)	2	225 (210-240)
Anesthetized						
SA	7	176 (115-205)	10	166 (120-230)	4	149 (80-220)
SA c partial AV block	0		0		2	79 (70-88)
SA c V Exs	2	153 (146-160)	0		1	150
AV	1	194	0		1	170
V	0		0		2	188 (185-190)
Recovered						
SA	2	127 (125-130)	4	139 (120-155)	4	129 (110-140)
SA c partial AV block	4	112 (100-125)	5	120 (92-140)	3	129 (112-140)
AV	1	152	0		2	180
Shifting pacemaker	1	195	1	140	0	
V	2	215 (190-240)	0		1	190

SA = Sino-auricular; AV = Auriculo-ventricular; V = Ventricular; V Exs = Ventricular extrasystoles; Shifting pacemaker = AV to V.

The increase of heart rate with cyclopropane in all dogs except No. 8 suggests a decreased vagal tone as postulated by Meek (5).<sup>§</sup> Such a reduction of vagal tone combined with an increased irritability of supraventricular tissues might be adequate to explain the counteraction of digitalis arrhythmias by cyclopropane.

5. Meek, W. J., Harvey Lectures, 1940-1941, Series XXXVI:188.

§ These results are in contrast to those observed by Rovenstine *et al.*(6) in isolated turtle hearts exposed to cyclopropane.

6. Rovenstine, E. A. and Adriani, *J. Anesth. and Analg.*, 1942, v21, 111.

All electrocardiograms taken during ether anesthesia showed either a return to normal conduction time or an upward displacement of the pacemaker of the over-digitalized heart. In only one dog was there failure to demonstrate an abnormal electrocardiogram upon recovery from anesthesia. Eight of the dogs had heart rate increases which ranged from 25 to 180%. One had no change and one a decrease of 18%.

The mechanisms responsible for the increased heart rate seen in ether anesthesia have been reported by Samaan(7) to be (a) paresis of the vagal inhibitory mechanism; (b) augmentation of the cardio-sympathetic

impulses; and (c) liberation of certain hormones such as epinephrine or sympathin. Paresis of the vagal inhibitory mechanism is directly antagonistic to one of the actions of digitalis. Decreased vagal stimulation of the SA and AV nodes would act to shift the pacemaker upward by virtue of the resultant greater automaticity of the nodal tissue. In 2 dogs, however, a slower rate was seen during anesthesia than was present in the unanesthetized but over-digitalized heart. An effect upon the relative irritability of the auricles and ventricles must be concluded, since the rhythm was SA with this slower rate.

With thiopental sodium 4 of the 10 animals had no change of the arrhythmia or conduction defect caused by over-digitalization; 2 were worse; and 4 were improved. In 3 of the latter 4 the short acting glycoside ouabaine had been used. Since electrocardiograms taken upon recovery from anesthesia did not show reversion to the abnormalities present before anesthesia, there is some doubt as to

7. Samaan, A., *Arch. internat. de pharmacodyn. et de therap.*, 1934, v50, 101.

whether the effect was due to the anesthetic agent or to the decrease in activity of the digitalis preparation.

Thiopental sodium was found to produce either no effect or an increase in responsiveness of the heart to vagal stimulation by Gruber *et al.*(8). In the present study this anesthetic agent was seen to exert no beneficial effect upon the severity of the arrhythmias induced by digitalis preparations.

*Summary.* Dogs, digitalized to cardiac toxicity with ouabaine (0.07 or 0.08 mg/kg) or with digitoxin (0.3 mg/kg) were anesthetized with cyclopropane, ether and thiopental sodium.

Cyclopropane improved or abolished the arrhythmias produced by digitalis preparations. Ether caused a reversion to normal rhythm. Thiopental sodium exerted no effect upon the electrocardiographic abnormalities of over-digitalized dogs.

8. Gruber, C. M., Haury, V. G., and Drake, M. E., *Am. Heart J.*, 1940, v20, 329.

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## Hydroxyproline Content of the Developing Chick Embryo. (18091)

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Previously reported investigations have shown that in the developing chick embryo the percentage of arginine and lysine remain quite constant, tyrosine and histidine decrease slightly, and tryptophan and cystine increase somewhat(1-4). Hydroxyproline, separated as the reineckate according to the procedure of Kapfhammer and Eck(5), has

been reported to occur in a constant amount in the egg during the period of incubation(6).

In the present study the colorimetric procedure of Neuman and Logan(7) for determining hydroxyproline shows it to be absent from egg white and yolk, and to accumulate in the embryo and embryonic membranes as development proceeds. The presence of hydroxyproline in the egg-shell membranes of the chicken, duck, and turkey is also reported.

6. Ido, R., *Ber. ges. Physiol. u. exp. Pharmakol.*, 1931, v63, 426.

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5. Kapfhammer, J., and Eck, R., *Z. physiol. Chem.*, 1927, v170, 294.

TABLE I. Hydroxyproline Content of the Egg.

Species	Hydroxyproline content of membranes—% of dry wt		
	Inner (egg)	Outer (shell)	Whole
Duck, White Pekin	1.12	0.66	0.89
Turkey, Mammoth Bronze	0.87	0.63	0.66
Chicken,* White Leghorn	1.12	0.89	1.00

\* Egg white and yolk and chalaza and vitelline membrane contained no hydroxyproline.

**Procedure.** White leghorn eggs were obtained from a commercial hatchery and incubated at 37.5 to 38.5°C at a humidity of 50 to 60%. They were turned once a day. The hydroxyproline content of white and yolk, chalaza and vitelline membrane, and egg-shell membranes of the fresh egg was determined by the colorimetric procedure of Neuman and Logan(7) with correction for color due to tyrosine. At intervals as the incubation proceeded, eggs were withdrawn and the embryos and embryonic membranes were removed, separated, blotted lightly with filter paper, and weighed. They were suspended in 20 to 50 times their weight of acetone for 3 hours, and overnight with the same quantity of fresh acetone, then removed and dried to constant weight at 108°C for analysis. To determine the extractable hydroxyproline, embryos and serosa were subjected to autoclaving in distilled water for two 3-hour periods at 15 lb pressure. The solutions and washings of the residues were transferred to test tubes. The contents were dried by directing a stream of air into the tube placed in a boiling water bath. The hydroxyproline content of the material removed by extraction was determined.

**Results.** As shown in Table I the whole egg-shell membranes from different species contained 0.66 to 1.00% hydroxyproline. The inner membranes of all species contained a higher content of hydroxyproline than did the outer membranes. The hydroxyproline was solubilized to an extent of 22% by autoclaving 6 hours at 15 lb. Pepsin and trypsin did not significantly digest the hydroxyproline-containing substances. Other keratinous materials analyzed by the present procedure did

not contain hydroxyproline(7). The egg white and yolk, and chalaza and vitelline membrane contained no detectable amounts of hydroxyproline.

As shown in Table II hydroxyproline was not detectable in the chick embryo during the first 4 days, but appeared in traces on the fifth day, and increased throughout the incubation period (19 days) to 1.16% of the dry weight. The logarithmic values of the total hydroxyproline of the embryo bear an approximately straight line relationship to the logarithmic values for the dry weight of the embryo. As recorded in Table II hydroxyproline of the serosa was not detectable until the sixth day. The content increased to a maximum of 2% on the fourteenth day. On the nineteenth day the total hydroxyproline of the extra-embryonic membranes was 0.92 mg and the total hydroxyproline of the embryo was 54.0 mg. The egg-shell membranes did not contribute significantly to the accumulated hydroxyproline for they did not de-

TABLE II. Hydroxyproline Content of Developing Chick Embryo and Extra-Embryonic Membranes.

Age of embryo, days	Dry wt of embryo, mg	Hydroxyproline content		Serosa, μg/mg
		Embryo μg/mg	Total mg	
1-4	5.6	0	0	0
5	5.8	.08	.0005	0
	7.1	.15	.0011	
6	7.3	.25	.0018	0.21
	11.3	.44	.0050	
7	39.6	1.14	.045	2.10
	53.0	1.46	.077	
8	55.2	1.77	.097	2.67
	61.9	1.69	.105	
9	63.9	1.95	.125	2.14
	112.8	3.01	.34	
10	134.6	3.78	.508	3.39
	150.4	3.33	.501	
11	163.1	3.67	.598	4.68
	250.0	4.80	1.20	
12	328.2	5.93	1.95	5.49
13	675.0	7.22	5.07	13.1
	700.4	6.42	4.52	
14	748.9	6.80	5.09	21.0
15	1645	7.45	12.02	16.5
16	2183	8.42	18.02	14.9
	2337	8.78	20.10	
17	2363	10.41	24.6	12.1
18	4552	11.15	50.8	12.0
19	4672	11.55	54.0	14.9
				12.0*

\* Amnion.

TABLE III. Extractable Hydroxyproline of Chick Embryo and Serosa.

Age of embryo, days	Dry wt of embryo, mg	Hydroxyproline		Determined, <sup>†</sup> μg/mg	Extracted, μg/mg
		Embryo	Serosa		
4	1.7	0	0		
10	100.0	3.00	3.05		
12	255.3	5.17	5.48		
13	701.3	7.06	7.50	10.8	10.6
15				16.5	15.4
16				8.6	9.2
17	3852	10.43	9.46	12.1	14.6
18				10.4	9.7
19				14.9	13.9

\* From best curve for log total hydroxyproline versus log dry weight of embryo.

† Serosa from the same egg as extracted serosa.

crease in weight or hydroxyproline content during incubation of the egg. The egg-shell membranes did not contain more than 1.7 mg of hydroxyproline.

Essentially the entire quantity of hydroxy-

proline-containing substances of the chick embryo and serosa at different ages is extractable by autoclaving (Table III). Apparently the hydroxyproline of these structures is principally in the form of collagen or collagen-like substances which are converted to gelatin by the autoclaving.

**Summary.** The egg-shell membranes of the chicken, duck, and turkey contain 0.66 to 1.00% hydroxyproline depending upon species. The remainder of the contents of the egg contains no detectable amount of hydroxyproline. Hydroxyproline appears in detectable amounts in the chick embryo after the fourth day and increases to 1.16% of the dry weight on the nineteenth day. The serosa contains hydroxyproline after the fifth day which increases to a maximum of 2.1% on the fourteenth day. Most of the hydroxyproline of the embryo and serosa is present in a form extractable by autoclaving, probably as collagen.

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### Inactivation of Microbiological Activity of Crystalline Vitamin B<sub>12</sub> by Reducing Agents.\* (18092)

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When crystalline vitamin B<sub>12</sub> is hydrogenated with platinum as catalyst, its red color disappears but can be restored upon shaking with air. In spite of the restoration of the original color, the product possesses only a small fraction of the original microbiological activity. Hence, it is biologically different and chemically distinct from vit. B<sub>12</sub> and is given the name vit. B<sub>12a</sub>(1). Since hydrogenation of vit. B<sub>12</sub> may involve a simple reduc-

tion (that is, gain of electrons) or addition of hydrogen atoms to double bonds, it is of interest to ascertain whether reducing agents can likewise abolish the microbiological activity of this vitamin. The results of such a study are presented in this communication.

**Experimental procedure and results.** A. *Inactivation of the microbiological activity of vitamin B<sub>12</sub> with various reducing agents.* Attempts were made to inactivate vit. B<sub>12</sub> with different reducing agents. The procedure of a typical experiment is given as follows: 400 mg of several reducing agents, such as cysteine hydrochloride, ascorbic acid, thiamine hydrochloride, and hydroquinone, were dissolved in 10 ml of water. One ml aliquots of these solutions were titrated with

\* The work was supported by the grants-in-aid from Hoffmann-La Roche, Squibb Institute for Medical Research, and Sharp and Dohme, Inc. The authors also appreciate the generous supply of vit. B<sub>12</sub> from Merck and Co.

1. Kaczka, E., Wolfe, D. E., and Folkers, K., J.A.C.S., 1949, v71, 1514.

TABLE I. Inactivation of Microbiological Activity of Vitamin B<sub>12</sub> by Various Reducing Agents at Different pH's.

Reducing agent added	pH			
	7.3	5.8	4.5	3.0
None (control)	0	0	0	0
Cysteine HCl	96	75	45	70
Thiamine HCl	91	25	28	0
Ascorbic acid	52	40	35	25
Hydroquinone	9	22	25	0

Inactivation is expressed as % of the original activity destroyed.

approximately 0.5 N NaOH to pH 7.3 in order to determine the amount of alkali necessary for neutralization. One-half ml of each of the solutions of reducing agents was pipetted into a series of tubes and neutralized with the requisite amount of sodium hydroxide. In order to stabilize the solution at the desired pH, 1 ml of 1.0 M phosphate buffer at pH 7.3 was added and the total volume of liquid in each tube was brought to 3.0 ml with water. Into each tube was pipetted 1.0 ml of a crystalline vitamin B<sub>12</sub> solution (200 µg/ml). For a control on the stability of this vitamin at pH 7.3, 1.0 ml of the vitamin solution was pipetted into a tube containing only 1.0 ml of phosphate buffer and 2 ml of water. To all tubes were added a few drops of toluene as a preservative. They were sealed and incubated at 37°C for 7 days, at the end of which the contents of the tube were diluted tenfold with water and assayed for the microbiological activity of vit. B<sub>12</sub>. The results are given in Table I. The data demonstrate that in the absence of a reducing agent there was no loss of microbiological activity at this pH, whereas in its presence the destruction was marked.

B. Effect of pH on inactivation. In order to ascertain whether hydrogen ion concentration plays a role in the inactivation of vitamin B<sub>12</sub> this vitamin was allowed to react with the same series of reducing agents at different pH's ranging from 4 to 7.5. Our other experiments showed that beyond this limit of hydrogen ion concentration, there was some appreciable loss of activity on incubation. The ratio of reducing agents to vit. B<sub>12</sub> remained 1 to 100 by weight. The results of this typical experiment (Table I)

demonstrate that under our experimental conditions there was no loss of microbiological activity in the control solutions kept at pH's between 7 and 4, whereas the destruction of microbiological activity by ascorbic acid, thiamine, and cysteine was most effective at the high pH (7.3). The inactivation by hydroquinone at pH 7.3 was less than that at pH 4.5 or 5.8. However, it should be pointed out that a considerable portion of the added reducing agent was destroyed at the neutral pH as evidenced by the appearance of the brownish color in the reaction mixture. The above data demonstrate that when vit. B<sub>12</sub> solution was allowed to react with a series of reducing agents, there was a destruction of microbiological activity. However, it was not accompanied by a concomitant decrease in the intensity of the red color. It is unlikely that the reduction of the microbiological activity is correlated to the disappearance of the red color. It is our belief that the inactivation is probably due to the reduction of the part of the vit. B<sub>12</sub> molecule which does not contribute to the color of the vitamin. The hypothesis that inactivation is due to simple reduction is further substantiated by the fact that methionine does not appreciably reduce the microbiological activity of vit. B<sub>12</sub>. This amino acid is similar to homocysteine except that the hydrogen of the SH group is replaced with a methyl group, thus making this compound incapable of reduction.

The animal protein factor activity of the treated substance was also tested by the use of vit. B<sub>12</sub> deficient rats. The procedure used was essentially that described by Register, *et al.*(2). The rats were born and raised by mothers on a soybean protein diet. A solution of vit. B<sub>12</sub> whose microbiological activity was destroyed by cysteine hydrochloride to the extent of 95% was tested for its APF activity at levels of 0.025 µg/day and 0.15 µg/day. The lower level was chosen because it represented the minimum dosage which will stimulate a definite growth response by our rats. The rats receiving the lower dosage gave as good a growth response

2. Register, U. D., Ruegamer, W. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1949, v177, 129.

as the rats in the control group which received an equal amount of untreated vitamin. Our data therefore indicate that if there were a simultaneous destruction of APF activity, the degree of destruction could not be as extensive as that of the microbiological activity.

*Summary and conclusion.* When a solution of crystalline vit. B<sub>12</sub> was subjected to a series of reducing agents between pH 4 and 7, at which range this vitamin is stable, a marked loss of the microbiological activity occurred. This loss of microbiological activ-

ity can be attributed to the reducing power of the agents, but is not necessarily related to the disappearance of the intensity of the red color. Our preliminary data also demonstrate that the destruction of the microbiological activity was not accompanied by the destruction to an equal extent of the APF activity.

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## Failure to Isolate Brucella from Prostatic Tissue of Individuals Living in an Endemic Area.\* (18093)

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Brucella have been recovered from the fallopian tubes, appendix, gall bladder, ovaries, serous surfaces of the intestines, joint fluid, feces and urine, and from extirpated human tonsils(1-4). Parsons and Poston(5) cultured brucella from the lymph nodes of 10 out of 19 cases of Hodgkins' disease, whereas brucella were recovered from the nodes of only one of 67 apparently normal individuals. Forbus and Gunter(6) reported that in 5 cases of Hodgkins' disease, *Brucella*

*suis* or *Brucella melitensis* was isolated from one or more of the following: lymph nodes, liver, spleen, testis, bile, kidneys, and rib marrow. More recently, McVay(7) cultured prostatic tissue obtained from 34 apparently healthy individuals. *Brucella abortus* was isolated from the glands of 2 men, and *Br. melitensis* from a third. This report has prompted a similar study at the University of Minnesota Hospitals. The majority of the patients entering the hospitals are referred from rural areas, where brucellosis is endemic. In a recent epidemiologic survey of bacteriologic proved brucellosis in Minnesota, it was observed that the disease not only occurred more frequently in a rural population, but also, that brucellosis was recognized most frequently in adult males(8). Through the cooperation and help of Dr. C. D. Creevy, Director of the Division of Urological Surgery at the University of Minnesota Hospitals, patients presenting themselves for resection of the prostate gland were selected for study.

\* Aided (in part) by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, United States Public Health Service; and by a grant for the study of brucellosis from the University of Minnesota.

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7. McVay, L. V., Guthrie, F., Michelson, I. D., and Sprunt, D. H., *PROC. SOC. EXP. BIOL. AND MED.*, 1948, v69, 607.

8. Magoffin, R. L., Kahler, P., Spink, W. W., and Fleming, D., *Pub. Health Rep.*, 1949, v64, 1021.

*Materials and methods.* Specimens of prostate gland removed by a transurethral approach and collected in sterile Petri dishes were ground in sterile saline solution. Three-fourths ml of the saline suspension of tissue was spread over the surface of plates of trypticase soy agar,<sup>†</sup> which contained gentian violet in a dilution of 1:700,000(9). The inoculated plates were incubated at 37°C in a jar containing 10% carbon dioxide, and observed for bacterial growth for 15 days before being discarded. Slide agglutination tests were carried out with suspected colonies and rabbit antibrucella serum. A potent antibrucella rabbit serum was prepared by immunizing with a strain of *Br. abortus* (Lynch 524). Identification of the bacteria, other than for brucella, was not attempted, except for studying smears prepared with Gram's stain. Two large loopfuls of the saline suspension of ground prostate gland were also inoculated into 2 ml of tryptose phosphate broth, and incubated at 37°C for 18 hours in a jar containing 10% carbon dioxide. If growth appeared, the organisms were exposed to the antibrucella rabbit serum for evidence of agglutination. Control cultural and serologic studies of the foregoing type were carried out simultaneously with a culture of *Br. abortus*. Suspensions of prostatic tissue were also inoculated into the yolk sacs of living chick embryos by a method utilized in this laboratory for culturing brucella and described elsewhere(10). This is a reliable method for recovering small numbers of brucella. Two-tenths ml of tissue suspension was injected into 5 day chick embryos. The eggs were incubated at 37°C for 2 to 7 days, and then loopfuls of yolk were transferred directly to plates of trypticase soy agar containing gentian violet, and the plates were placed in a jar containing 10% carbon dioxide and incubation was carried out at 37°C for 15 to 20 days. Control experiments indicated that an inoculum of as

few as 150 colonies of *Br. abortus* in prostatic tissues could be recovered from the eggs. Brucella were separated from other bacterial contaminants by the method of Amoss(2).

As an indication of the exposure of the patients to brucellosis, intradermal tests were carried out with brucellergen(9). A positive test represented a local reaction with 1 cm or more of edema 48 hours after injecting the antigen. Agglutination tests on the serum of the patients were performed with an antigen of *Br. abortus* obtained from the Bureau of Animal Industry, of the United States Department of Agriculture. The tube agglutination test was done with falling concentrations of serum, and evidence of agglutination was noted at the end of 24 and 48 hours of incubation at 37°C in a water bath.

*Results.* Prostatic tissue was obtained from 100 males. The ages of the patients varied from 50 to 90 years. The vast majority were farmers, and 10 stated that they had been exposed to Bangs' disease in their cattle. In 85 patients, the histologic diagnosis of the excised prostatic tissue was benign prostatic hypertrophy. The remaining 15 patients had carcinoma of the prostate. Except for the first 16 patients, suspensions of prostatic tissue were injected into chick embryos, and in not a single instance was brucella recovered. Prostatic tissue from every patient was cultured for brucella in tryptose phosphate broth and the tissues were also inoculated on plates of trypticase soy-gentian violet agar. Brucella were not isolated from any of the material. That many of the patients had been exposed to brucellosis was revealed by the fact that 29 patients or almost one-third of those tested had positive skins for brucellergen. Furthermore, 38 or approximately one-third of the patients had agglutinins for brucella in their sera. In 24 of these patients, the agglutinin titer was 1:80 or less. In 14 patients the titer was 1:160 or over. Two individuals had titers of 1:1280. While this information corroborated a history of exposure to the disease, in no instance was there definite evidence of an active infection being present.

<sup>†</sup> Baltimore Biological Laboratory.

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**Summary.** 1. Prostatic tissue obtained surgically from 100 consecutive males, who had lived for many years in an endemic area of brucellosis in Minnesota, were cultured for brucella and in no instance were brucella isolated. 2. On the basis of historical evi-

dence, as well as positive intradermal tests and the presence of brucella agglutinins, many of the individuals had been exposed to brucellosis.

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### Reversal of Aminopterin Inhibition in the Chick Embryo with the *Leuconostoc citrovorum* Factor.\* (18094)

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In an earlier study(1), minute amounts of aminopterin were shown to inhibit development of the chick embryo. Under the conditions tested, this inhibition could be partially alleviated by thymidine, and more effectively by thymidine plus hypoxanthine desoxyriboside but not by folic acid, vitamin B<sub>12</sub> or several other substances. The conclusion was drawn that aminopterin inhibited synthesis of thymidine and one or more of the purine desoxyribosides by the embryo, and that by eliminating the need for these synthetic reactions during the early stages of embryonic development, added desoxyribosides counteracted the inhibitory action of aminopterin. Since aminopterin appears to act as an anti-folic acid, the direct or indirect participation of folic acid in synthesis of these desoxyribosides was indicated. Recent evidence(2-6) indicates that the *Leuconostoc citrovorum* factor (CF) is a metabolically active form of folic acid, and that

it can be formed synthetically from folic acid(5,6). Concentrates of this substance counteract aminopterin inhibition of *Leuconostoc citrovorum*(4). Amounts of CF previously available for testing failed to alleviate aminopterin inhibition of the chick embryo; it was pointed out, however, that larger amounts might prove effective(1). It is shown below that adequate amounts of CF partially counteract aminopterin inhibition of the chick embryo under conditions where folic acid is ineffective.

**Experimental.** Procedures used in injecting solutions and incubating eggs were exactly similar to those described previously(1,7). All injections made prior to incubation totaled 0.1 ml. Those made at 3 days totaled 0.2 ml.

**Results.** Table I presents the results obtained when aminopterin was injected alone or together with CF concentrate, folinic acid (6), or folic acid just prior to incubation. A pronounced effect of CF in reversing the inhibitory effect of aminopterin is evidenced not only by the increased hatch obtained in lots receiving adequate amounts of this substance, but by the greatly decreased number of embryos that died early in the incubation period. Larger amounts of the concentrate are required for this purpose in those lots receiving 20 µg than in those receiving 10

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

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TABLE I. Counteraction of Aminopterin Inhibition of Chick Embryo by CF.  
Injections at zero days incubation.

Substance inj., amt	No. of eggs	% embryos dying at		% hatch
		0-5 days	6-20 days	
None	19			100
Water (0.1 ml)	20	5.0	15	80
Aminopterin, 20 µg	19	89.5	10.5	
" 20 µg				
+ CF,* 300,000 units	10	100		
+ CF, 600,000 "	20	85		15
+ CF, 1,200,000 "	20	65	10	25
+ CF, 3,000,000 "	20	35	30	35
Aminopterin, 10 µg	10	90		10
" 10 µg				
+ CF, 600,000 units	10	90		10
+ CF, 1,200,000 "	10	50		50
+ CF, 3,000,000 "	10	20	20	60
+ Folinic acid,† 10 µg	8	62.5		37.5
+ " " 500 µg	10	100		

\* The CF concentrate used was kindly supplied by Drs. T. H. Jukes and H. P. Broquist of Lederle Laboratories. It contained 30,000,000 CF units per ml; and 300,000 units per mg of solids. One CF unit is the amount required per ml of medium to permit half maximum growth of *Leuconostoc citrovorum* under defined conditions(2).

† The sample of folinic acid, kindly supplied by Dr. W. Shive, was synthesized from folic acid(6) and subsequently fractionated until free of folic acid and of formylfolic acid (personal communication). This product promotes growth of *Leuconostoc citrovorum* under the same conditions as CF, and it appears likely that the two products are identical(6,8). The absolute purity of the product tested is not known.

µg of aminopterin. In confirmation of previous results(1) folic acid was ineffective in alleviating the inhibition when injected at zero days.

Assay with *Lactobacillus delbrueckii* 730, an organism that requires thymidine for growth(9,10), showed the CF concentrate to be free of this desoxyriboside. Its effects thus cannot be ascribed to this substance or to folic acid. The limited amount of folinic acid available showed definite activity in counteracting aminopterin inhibition, and since this substance was prepared synthetically from folic acid and has high CF activity, the evidence is conclusive that the favorable effects of the CF concentrate are actually due to the CF that it contains.

The effects on hatchability of similar injections made after 3 days of incubation are shown in Table II. The effect of CF in overcoming the toxic effects of aminopterin is unmistakable. Folic acid and formylfolic acid appear to be slightly effective under

TABLE II. Effect of Aminopterin and Related Compounds on Development of 3-Day Embryos.

Substance inj., amt	No. of eggs inj.	% hatch
None	10	100
Water, 0.2 ml	18	94.5
Aminopterin, 20 µg	19	21
" 20 µg		
+ CF, 600,000 units	10	30
+ CF, 1,200,000 "	10	50
+ CF, 3,000,000 "	17	76.4
+ CF, 6,000,000 "	10	70
+ Folic acid, 500 µg	18	33.3
+ Formylfolic acid, 500 µg	10	40

these conditions, but much less so than CF.

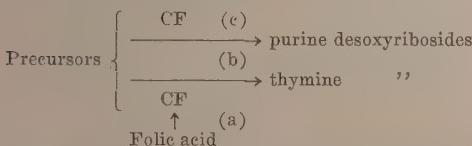
*Discussion.* It was observed previously that mixtures of thymidine and hypoxanthine desoxyriboside partially counteracted the inhibitory effects of aminopterin on growth of the chick embryo under conditions where folic acid was ineffective. The present data demonstrate that similar reversals are effected by the *Leuconostoc citrovorum* factor (CF), a substance which is derived from folic acid in intact animals(11), in liver slices(5), and by non-enzymatic, chemical

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means *in vitro*(6). These data are most readily explained by assuming (a) that conversion of folic acid to CF is a necessary preliminary step to its participation in catalysis of certain synthetic reactions, (b) that among the synthetic reactions for which CF is essential are syntheses of thymidine and one or more of the purine desoxyribosides, and (c) that the latter reactions are the first to be inhibited by aminopterin in the developing embryo. These postulates are expressed diagrammatically as follows:



The arrows may represent more than a single chemical transformation.

The growth-promoting action of CF (or folinic acid) for organisms which require folic acid(8) is thus readily explained, since these organisms must normally convert folic acid to CF before it can be utilized. Existence of organisms such as *L. citrovorum*, which cannot utilize folic acid readily in place of CF is predicted by this scheme; such organisms fail to carry out conversion (a) readily. The scheme also explains the enhanced activity of CF, as compared with folic acid, in counteracting growth inhibition by methylfolic acid(3) and by aminopterin (cf. 4, 12, and this paper); for precursors of a metabolite are frequently much less effective than the metabolite itself in counteracting the inhibitory effects of an antimetabolite(13,14). Finally, the scheme adequately accounts for reversal of aminopterin inhibition by thymidine or by thymidine and hypoxanthine desoxyriboside, observed both in bacteria(4,15) and the chick embryo(1); for aminopterin, at the concentrations used, is visualized as blocking primarily reactions (b) or (b) and (c). When the desoxyribo-

sides are supplied preformed, the blocked reactions are no longer required for growth.

The relative activity of CF in reversing the toxic effects of aminopterin for various organisms varies widely. For *L. citrovorum*, 11 units of CF counteracted the toxic effects of 1-4  $\mu\text{g}$  of aminopterin(4); in mice, about 20,000 units of CF was required for this purpose†(12), while the corresponding figure for the chick embryo is 100,000-200,000 units or more. The best estimates available indicate that 1 unit of CF is equivalent to 0.1-0.2  $\mu\text{g}$  of folic acid in growth-promoting activity for *Streptococcus faecalis*.‡ Assuming the two products to have equimolar activities and approximately equal molecular weights, 200,000 units of CF would be equivalent to about 20  $\mu\text{g}$  of the pure vitamin.

**Summary.** Concentrates of the *Leuconostoc citrovorum* factor (CF) and of "folinic acid" partially counteract the inhibitory action of aminopterin for the chick embryo. Folic acid and formylfolic acid were ineffective or only slightly effective under the same conditions. The magnitude of the effects obtained with CF were similar to those obtained previously with mixtures of thymidine and hypoxanthine desoxyriboside(1). The viewpoint is developed that conversion of folic acid to CF is a necessary preliminary to the catalytic action of the former compound, that synthesis of thymidine and one or more of the purine desoxyribosides are among the synthetic reactions for which CF (or folic acid) is required, and that these synthetic reactions are those inhibited in the chick embryo by small amounts of aminopterin. The great variation that exists in the ability of CF to counteract aminopterin inhibition in various organisms is pointed out.

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## Some Effects of Continued Protein Deprivation, with and without Methionine Supplementation, on Intracellular Liver Components.\* (18095)

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It has been reported previously from this laboratory(1) that dietary protein deprivation of rats for a 3 week period causes marked changes in the contents of nitrogen, pentose nucleic acid (PNA), and desoxypentenosenucleic acid (DNA) of the various intracellular components of their livers. Since an extension of the period of dietary protein restriction results in certain compensatory changes in the liver which make its composition resemble more nearly the normal(2,3), the effects of 5 week protein depletion on the morphological constituents of the liver were studied. In addition, because methionine has been shown to exert a protective effect upon the liver under various conditions(4), the influence on the intracellular liver components of rats on a protein-free diet supplemented with this amino acid was investigated.

*Experimental.* Forty-two female rats of the Wistar strain, between 12 and 18 weeks of age, and weighing between 166 and 210 g, were placed on a normal diet, adequate in all respects, for a period of 7 days. At the end of this time the animals were divided into 3 groups of 14. Group I was placed on a protein-free diet for a period of 5 weeks. Group II was maintained on a protein-free diet containing 1% DL-methionine for the 35 day period. The last group served as controls and was continued on the normal diet for the same period of time.

The normal and protein-free diets were

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3. Wang, C., Hegsted, D. M., Lapi, A., Zamcheck, N., and Black, M. B., *J. Lab. Clin. Med.*, 1949, v34, 953.

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identical in composition with the corresponding diets reported in a previous study(1). The methionine-supplemented diet was similar to the protein-free diet except that it contained 1% DL-methionine which replaced a corresponding amount of glucose. The livers of the animals were obtained and prepared for analysis as described previously(1). Water and fat were determined as before, and glycogen was determined by the anthrone method(5). A 5 ml aliquot of a 10% homogenate of the liver was fractionated into so-called nuclear, mitochondrial, microsomatic, and residual cytoplasmic fractions by the differential centrifugation method of Schneider(6), and the fractions as well as the original homogenate were analyzed for nitrogen, DNA, and PNA. Nitrogen was determined by the micro-Kjeldahl procedure, the ammonia formed being steam-distilled and titrated. The DNA and PNA were determined by a modification of the method described by Ogur and Rosen(7), which is based upon the observation that cold perchloric acid extracts PNA only, whereas hot perchloric acid extracts both PNA and DNA. The essential steps of the procedure are detailed below. Each of the sedimented fractions is diluted to 10 ml with isotonic sucrose solution, and a 1 ml portion precipitated with 2.5 ml of cold 10% trichloroacetic acid. The centrifuged precipitate is then washed successively with additional amounts of trichloroacetic acid, with 70% ethanol containing 0.1% perchloric acid (PCA), then treated at 50°C for 1 minute with a 3:1 ethanol-ether mixture, and further washed with 2% PCA. Centri-

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fugation following each treatment is carried out in the cold except after the alcohol-ether extraction when the operation is performed at room temperature. The further treatment of the washed residue depends upon whether the fraction contains only PNA or both PNA and DNA.

*For fractions containing only PNA (mitochondrial, microsomatic, and residual cytoplasmic):* The residue is extracted 3 times with 10% PCA at 80°C for 20 minutes, and the extracts are combined and diluted to 10 ml with 10% PCA. *For "fractions" containing both PNA and DNA (nuclear fraction and homogenate):* The residue, suspended in 10% PCA, is allowed to stand at 4°C for 18 hours, centrifuged, and washed twice by centrifugation with cold 10% PCA. The washings and original extract are combined and diluted to 10 ml with 10% PCA. The extract obtained in this manner contains PNA only.

For the extraction of DNA, the residue remaining after the extraction of PNA is treated as described above for fractions containing only PNA. Thus a second extract is obtained containing only DNA. The PNA or DNA contents of the various extracts were determined spectrophotometrically as described by Ogur and Rosen(7). The results obtained by this method for the PNA contents of liver homogenates and the various sedimented fractions agreed with the values obtained by the orcinol method(8) applied to trichloroacetic acid extracts(9) of the same liver samples. The DNA contents of liver homogenates as determined from the spectral absorption of PCA extracts were slightly higher than the values obtained on trichloroacetic acid extracts from the same homogenates employing the diphenylamine method (10). When applied to the nuclear fraction for the determination of DNA, the method of PCA extraction and spectrophotometric measurement gave exceedingly low values.

Thus, the amount of the total liver DNA recovered in this fraction varied between 60 and 80%. It is not likely that this discrepancy is a result of partial extraction of the DNA during the extended treatment of the fraction with PCA in the cold, inasmuch as this would result in an abnormally large value for PNA in the nuclear fraction. In all cases, however, the nuclear PNA values compared favorably with values obtained by other methods.

*Results.* The animals maintained on the control diet for 5 weeks gained on the average 5.8 g per week. The animals subsisting on the protein-free diet and those on the protein-free, methionine-supplemented diet for this period of time lost on the average 9.8 g and 9.4 g per week respectively. The average liver weights at sacrifice of the various groups of animals were as follows: control, 7.3 g; protein-free, 5.1 g; and protein-free, methionine-supplemented, 5.2 g.

The results obtained for liver, fat, water, and glycogen on the control animals were similar to those reported previously(11). The livers of Group I animals showed no change in water content as compared with the controls. The fat content of the liver, however, increased from the control value of 26.0 to 77.8 g per kg for the protein-free group, and the glycogen content of the liver increased

TABLE I. Desoxypentenucleic Acid Content of Liver Cell Homogenates from Control and Protein-deficient Rats.

	mg per g liver	Avg	$\sigma$ †	<i>t</i> value*
Control		3.52	0.40	
,, N	115		10	
Protein-deficient		4.08	0.37	3.62
,, N	718		19	10.95
Protein-deficient + methionine		4.01	0.29	3.70
Protein-deficient + methionine N	170		14	9.78

\* The *t* value is the expression of the significance of the difference between the means. A figure of 3 or greater is of definite significance (Hill, A. B., Principles of Medical Statistics, (1942), London, 3rd edition).

†  $\sigma$  represents the standard deviation.

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TABLE II. Nitrogen Contents of Liver Cell Fractions from Control and Protein-deficient Rats.

	Control		Protein-deficient			Protein-deficient + methionine		
	Avg	$\sigma$	Avg	$\sigma$	t value	Avg	$\sigma$	t value
mg per g liver	30.77	1.23	Homogenate					
			23.00	1.61	14.26	23.63	1.94	11.60
mg per g liver	6.55	0.38	Nuclear fraction					
% of total	21.3	1.1	6.32	0.58	1.16	6.73	0.74	0.78
			27.7	2.3	8.75	28.3	1.6	13.00
mg per g liver	5.51	0.73	Mitochondrial fraction					
% of total	18.0	2.3	3.23	0.41	9.72	3.48	0.64	7.38
			14.1	1.8	4.71	14.7	2.3	3.67
mg per g liver	6.71	0.50	Microsomatic fraction					
% of total	21.2	2.4	4.42	0.45	11.86	4.44	0.34	13.62
			19.3	2.1	2.07	18.8	1.4	3.12
mg per g liver	12.64	0.39	Residual cytoplasmic fraction					
% of total	41.3	1.3	9.99	0.98	8.72	9.96	0.77	11.15
			43.7	1.3	4.62	42.1	2.5	1.03
% recovery	102.6	3.9		104.8	2.9		103.8	4.3

TABLE III. Pentose nucleic Acid Contents of Liver Cell Fractions from Control and Protein-deficient Rats.

	Control		Protein-deficient			Protein-deficient + methionine		
	Avg	$\sigma$	Avg	$\sigma$	t value	Avg	$\sigma$	t value
mg per g liver	7.43	0.33	Homogenate					
" " " " N	242	18	305	28	2.02	7.27	0.83	0.07
					6.88	308	24	8.25
mg per g liver	1.15	0.11	Nuclear fraction					
% of total	15.4	1.4	1.34	0.17	3.38	1.40	0.25	3.30
mg per g fraction N	176	17	19.1	2.7	4.43	19.3	2.2	5.33
			213	25	4.17	208	18	4.57
mg per g liver	0.60	0.14	Mitochondrial fraction					
% of total	8.0	1.6	0.58	0.15	0.34	0.55	0.04	1.19
mg per g fraction N	106	18	8.2	1.76	0.30	7.7	2.1	0.41
			181	44	5.53	163	42	4.51
mg per g liver	3.75	0.53	Microsomatic fraction					
% of total	50.4	4.9	2.84	0.38	4.96	2.92	0.31	4.87
mg per g fraction N	560	47	40.3	5.7	4.78	40.5	5.2	5.03
			646	53	4.15	656	59	4.58
mg per g liver	2.57	0.25	Residual cytoplasmic fraction					
% of total	34.4	3.0	3.12	0.34	4.58	2.86	0.44	2.07
mg per g fraction N	204	20	44.3	4.0	6.85	39.4	4.2	3.46
			313	21	13.33	286	33	7.57
% recovery	108.2	6.5		111.9	6.1		106.9	7.0

from 6.16 to 7.50%. The analysis of the livers of the Group II rats for these constituents yielded results not appreciably different from those obtained on the livers of Group I animals.

Table I summarizes the results of the analyses of the liver homogenates for DNA, while those obtained for the nitrogen and PNA contents of the liver homogenates and the various intracellular fractions are presented

in Tables II and III. At the outset it should be noted that on the whole the results obtained with the methionine-supplemented animals were similar to those obtained with the protein-free group.

The liver homogenates of Group I animals showed a decrease in nitrogen, an increase in DNA, and no significant change in PNA as compared with the control group.

The nuclear fractions from livers of Group I animals showed no significant change in nitrogen content but contained significantly more PNA than did the corresponding fractions from control animals. On the other hand, there was a marked loss in nitrogen from the mitochondrial fractions of Group I livers as compared with the controls, and there was no significant change in the PNA values.

The results further show that a loss of PNA from the microsomatic fractions of Group I animals was accompanied by an increase of this constituent in the residual cytoplasmic fractions of the livers. Although there was a comparable loss of PNA from the microsomatic fractions of Group II animals, the gain of PNA by the residual cytoplasmic fractions of these same animals was not so great in magnitude as was encountered in the case of Group I animals.

Finally, there was a significant loss of nitrogen from the liver microsomatic fractions of both Groups I and II which was accompanied by a decrease in the absolute amount of nitrogen in the residual cytoplasm. However, there was a significant increase in the percentage of the total nitrogen located in the latter fraction in the case of the Group I animals which was not encountered in the case of the Group II rats.

**Discussion.** The animals maintained on a protein-free diet for 5 weeks showed a 25.2% decrease in liver nitrogen when compared to control animals, whereas animals on a similar diet for 3 weeks showed a 19.3% decrease in liver nitrogen. As in the case of the 3 week animals, the loss in liver nitrogen by the 5 week group could be accounted for by a loss of nitrogen from the mitochondrial, microsomatic, and residual cytoplasmic fractions, while the nuclear fraction was rela-

tively unaffected. In the case of the 5 week animals, however, the loss of nitrogen tended to be more equally divided among the 3 affected fractions than in the case of the 3 week animals. However, the loss of nitrogen was greatest from the microsomatic fraction in the latter group, whereas in the 5 week animals the loss was greatest from the residual cytoplasm. For the present, the significance of this difference between the 2 groups of animals is not apparent. As was observed with the 3 week animals, the livers of the 5 week protein-deficient rats showed an increase of DNA. In the latter instance, however, the change was of unquestionable statistical significance. This increase in DNA can be interpreted as the result of an increased number of cells per unit of liver or an elevated concentration of DNA per cell nucleus. It has been shown that in other experimental situations resulting in an elevated liver DNA, the increase of this constituent can be accounted for by an increased cell density(12,13), an explanation which probably applies to conditions of protein-deficiency as well.

Although livers from the rats subsisting on the protein-free diet for the 5 week period contained slightly less PNA than did the corresponding control livers, this difference was only of probable significance. However, while the total PNA change was not marked, there was a striking redistribution of this constituent among the several cellular fractions when compared with the controls. Thus, an increased amount of PNA was present in the nuclear fraction of Group I animals, while there was a marked decrease in the PNA of the microsomatic fraction and an increase of the PNA in the residual cytoplasm. Such a redistribution of the PNA between the latter 2 fractions was previously described for 3 week protein-deficient animals(1), but the present data make it apparent that extended protein depletion magnifies this "shift." Recently, in a brief report on the effects of

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a 40 day protein-fast on the liver PNA, Vendrely and Vendrely(14) came to the conclusion that the loss of this constituent (which is apparent only when the values are expressed in terms of "initial liver weight") stems almost exclusively from the microsomes. The results reported in the present paper are generally in accord with those of the French authors inasmuch as the greatest change in PNA content occurred in the microsomatic fraction, but the interpretation presented here places the emphasis on the redistribution of PNA between this fraction and the residual cytoplasm rather than on the absolute loss from the cells.

It is important to point out that the encountered changes in the distribution of PNA and nitrogen among the various fractions seem to fall into a general pattern similar to that which has been reported by Price and co-workers(15-19), and by Cunningham, Griffin, and Luck(20) for cell fractions obtained from hepatoma tissue *per se* and from livers of rats maintained on high or low protein diets containing active carcinogenic dyes. Thus these workers have reported that hepatomas contained more DNA and nitrogen in the nuclear fraction, less PNA and nitrogen in the mitochondrial and microsomatic fractions, and considerably more PNA in the residual cytoplasm than did normal livers. Similar changes from normal were reported for liver fractions from rats fed active carcinogenic compounds although the magnitude of the changes was not so great. In the case of protein-deficient animals the livers con-

tained more DNA and PNA, but an equal amount of nitrogen in the nuclear fraction, less nitrogen but an equal amount of PNA in the mitochondrial fraction, less PNA and nitrogen in the microsomatic fraction, and considerably more PNA in the residual cytoplasm than did the livers of control animals. The extent of these changes was related to the period of time that the animals were kept on the protein-free diet. It would appear, therefore, that the pattern of PNA and nitrogen distribution just described is more general than previously believed(19), and cannot be specifically related to existence of a pre-cancerous condition.

The only apparent difference between the results obtained with animals on the protein-free diet and those on the same diet supplemented with 1% methionine was the obviation of the increase in the PNA content of the residual cytoplasm. The significance of this effect of methionine is not at present understood.

**Summary.** The intracellular distribution of nitrogen and nucleic acids in the livers of rats fed a diet free of protein for 5 weeks was found to be similar to that previously observed in rats subsisting on the same diet for 3 weeks, although the changes from control values were of greater magnitude in the case of the animals depleted for the longer period of time.

The inclusion of 1% DL-methionine in the protein-free diet had no appreciable effect in protecting the liver from the observed changes due to protein depletion except in partially preventing the increase of PNA in the residual cytoplasmic fraction. It is noted that the changes encountered in the intracellular distribution of nitrogen and nucleic acids in the livers of protein-deficient animals fall into the same pattern as the changes which have been found to obtain in the livers of pre-cancerous and cancerous animals.

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## Prevention of Pyrogen-Induced Renal Hyperemia in the Dog by Dihydroergocornine.\* (18096)

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Although the renal hyperemia resulting from the administration of bacterial pyrogens is not prevented by sympathectomy or "virtual denervation" of the kidney(1,2), and is not prevented by antipyretic drugs(3) the possibility remains that the increased blood flow results indirectly from an action of the pyrogens on the central nervous system. Since dihydroergocornine (DHO) is known to interfere centrally with certain autonomic mechanisms(4,5), the experiments reported below were undertaken to determine the effect of this agent on the response of the renal vascular bed to pyrogens.

**Methods.** Experiments were conducted on trained female dogs without anesthesia or sedation. Glomerular filtration rate and effective renal plasma flow were estimated from creatinine and para-aminohippurate clearances. After a priming dose, these substances were infused at a constant rate in pyrogen-free 5% glucose solution through an intravenous catheter. Urine was collected from a catheter in periods of 15 minutes; the bladder was washed with three 15 cc portions of water and two 20 cc portions of air at the end of each period. Most animals were subjected to "control" runs in which pyrogen alone (2.5 γ total dose) was administered, followed by or preceded by "experimental" runs in which pyrogen was administered 30

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minutes after the injection of DHO in a dose of .01 mg per kg intravenously plus .01 mg per kg subcutaneously.‡ Successive experiments on the same animal were separated by an interval of at least 10 days. Rectal temperatures were recorded in all experiments.

In every experiment 2 or 3 control periods were run on each animal to determine the basic glomerular filtration rate and effective renal plasma flow levels. A pyrogen, derived from the capsule of one of the Shigella group of organisms,§ was then injected intravenously in a fixed dose of 2.5 γ per dog. Samples of urine and blood were taken for 6 or 7 additional periods.

**Results.** In confirmation of numerous previous investigations, it was found that the pyrogen increased renal plasma flow in every animal. The average of maximum responses was 44%, with a range of 27% to 79% (Table I). The renal blood flow increased after a latent period of 30 to 45 minutes, and was usually still elevated at the time collections were discontinued.

No significant changes in glomerular filtration rate were observed, and the creatinine clearance data are consequently omitted from the table.

Rectal temperatures rose in every case, even though some of the animals were hyperthermic at the outset because of high environmental temperatures.

In contrast to the effects of pyrogen in untreated animals, the maximum hyperemic responses after premedication with DHO were greatly diminished (Table I, Fig. 1). The average of maximum responses in 7 animals was +10%, with a range from -4% to +25%. DHO did not alter glomerular filtra-

‡ DHO-180 was generously supplied by Sandoz Pharmaceuticals.

§ The pyrogen was prepared and generously supplied by Dr. Herbert Morgan of the University of Michigan School of Public Health.

TABLE I. Effect of Pyrogen on Renal Plasma Flow With and Without DHO Pre-medication.

Dog	Pyrogen alone			DHO-180 + pyrogen		
	R.P.F.		Change, %	R.P.F.		Change, %
	Control, cc/min.	Pyrogen, cc/min.		Control, cc/min.	Pyrogen, cc/min.	
1	200	358	+79	191	239	+25
10	149	226	+52	181	190	+5
13	158	227	+44	134	160	+19
16	175	232	+33	128	123	-4
9	117	148	+27	—	—	—
19	—	—	—	141	147	+4
25	176	239	+36	118	113	0
26	165	228	+38	105	124	+18
Avg (S.E. = 6.5; p = <.001)			+44.1	Avg (S.E. = 4.2; p = <.1, >0.05)		
						+ 9.6

tion rate in these experiments, nor did it prevent the hyperthermic response to pyrogen.

**Discussion.** Though DHO has no significant action on renal plasma flow in the normal or denervated kidney(6), it has been shown to abolish the renal vasoconstriction induced by epinephrine in the dog. This

presumably results from the peripheral "adrenolytic" action of the alkaloid, which action is particularly prominent in the renal vascular tree. The effect of DHO in preventing the renal hyperemia induced by pyrogens can hardly be an adrenolytic action, unless it be assumed that in response to pyrogens adrenergic vasodilatation can occur in the kidney, and that this dilatation can also be prevented by an adrenolytic drug. This seems to us to be an unlikely hypothesis, and it seems more probable that the antagonism between DHO and the pyrogen is a manifestation of a blocking action of the alkaloid in the central nervous system. The data presented in this study do not, of course, prove such a concept, and a peripheral antagonism in the renal vessels remains a possibility.

**Summary.** Premedication with moderate doses of dihydroergocornine greatly diminished the renal hyperemia produced in the dog by a bacterial pyrogen. No significant change of glomerular filtration rate was observed following administration of either the pyrogen or the alkaloid.

Response of renal plasma flow to *Shigella pyogenes*, with and without premedication with dihydroergocornine.

Para-aminohippurate clearance (P.A.H.) and creatinine clearance (Creat.), scale at left in cc/min. Rectal temperature, scale at right. Time in minutes on the abscissa. Dog. #13.

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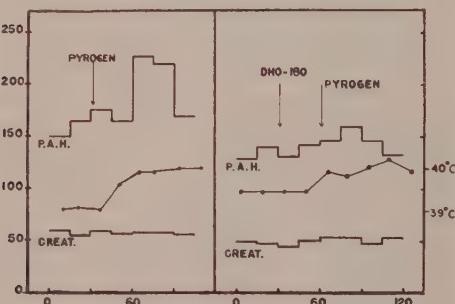


FIG. 1.

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## Effect of Phthalylsulfathiazole on Growth and Reproduction of Rats Fed a Soybean Protein Ration.\* (18097)

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The possibility of an improved performance of animals consuming bacteriostatic or bactericidal agents was clearly visualized and expressed by Moore *et al.*(1). They observed increased weight gains of chicks when streptomycin or sulfasuxidine were added to a purified ration. More recently, Stokstad and Jukes(2) found that the feeding of aureomycin to chickens stimulated their growth. Similarly, marked increases in weight gains of pigs fed plant rations have been reported as a result of the addition of aureomycin(3,4) and of streptomycin(5) to these rations. While the reasons for these effects of the antibiotics are at present largely a matter of conjecture it is quite possible that their beneficial effects are intimately related to their action on the microflora of the intestinal tract.

Miller(6) and Kon(7) have reviewed much of the evidence which supports the view that insoluble sulfonamides, including phthalylsulfathiazole (PST) exert their effect on the animal organism primarily through modification of the intestinal flora. A temporary(8)

\* This is the fourth of a series of papers dealing with the nutritional value of plant materials. Paper No. 2429, Scientific Journal Series, Minnesota Agricultural Experiment Station.

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2. Stokstad, E. L. R., and Jukes, T. H., *PROC. SOC. EXP. BIOL. AND MED.*, 1950, v73, 523.

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5. Luecke, R. W., McMillan, W. N. and Thorp, F., Jr., *Arch. Biochem.*, 1950, v26, 326.

6. Miller, A. K., *J. Nutr.*, 1945, v39, 143.

7. Kon, S. K., *Proc. Nutr. Soc.*, 1945, v3, 217.

8. Gant, O. K., Ransone, B., McCoy, E., and Elvehjem, C. A., *PROC. SOC. EXP. BIOL. AND MED.*, 1943, v52, 276.

or more permanent(6,9) depression of the number of coliform organisms in the intestine can apparently be compensated by an increase in other species so that the total bacterial count may not be reduced(6,8). The depression of growth(10), the granulocytopenia and leucopenia(11), the interference with reproduction(12) and lactation of rats(13) caused by sulfonamides can be overcome by feeding sufficient quantities of folic acid with the diet. With an adequate intake of folic acid and biotin the growth(10,14,15) and reproductive performance of rats are not impaired(16). In fact, improved growth performance of rats maintained on adequate natural or purified diets containing insoluble sulfonamides has been observed(10,15,17). However, Jones *et al.*(18) reported that the growth depressing effects on rats fed rations containing 5% of sulfasuxidine could not be completely overcome by folic acid unless liver extract was also supplied. Recent observations made in this laboratory in connection with another study(19) provide evidence for the favorable effect of a bacteriostatic compound on the nutrition of a mammalian species. In the experiment re-

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17. Baxter, J. H., *J. Nutr.*, 1947, v34, 333.

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19. Schultze, M. O., *J. Nutr.*, 1950, v41, 103.

## GROWTH AND REPRODUCTION OF RATS

TABLE I. Effect of Phthaloylsulfathiazole on Growth, Reproduction, and Acute Uremia of the Newborn.

	Basal ration	Basal ration + 2% PST
No. of female rats	15	15
6 weeks post weaning wt gain (g)	109.9 ± 4.9*	114.1 ± 3.5*
No. of sterile females	0	1
No. of pregnancies	38†	26†
No. of litters born alive	37	26
Mean No. of young born per litter	7.03 ± 0.40*	7.65 ± 0.59*
% of young born dead	5.0	1.0
Mortality of young 0-4th day %	37.2	6.1
Mortality of young 0-21st day %	53.4	32.9
Incidence of acute uremia of newborn; % of litters	85.1	0
Mean No. of young weaned per litter survivors	5.2 ± 0.49*	5.7 ± 0.51*
Mean 21 day wt per survivor (g)	34.1 ± 0.42*	36.5 ± 0.45*
Litters with 6 or more young at 21 days	13	11
Mean No. of young in these litters	6.8	8.2
Mean wt change of mothers during lactation (g)	-1.2 ± 3.0*	+14.6 ± 3.3*

\* Standard error of the mean.

† The larger number of pregnancies on the basal ration is due to the fact that 14 litters died within 96 hours after birth and their mothers were immediately bred again.

ported in the present paper phthalylsulfathiazole (PST) was fed to rats maintained on a ration containing a commercial soybean protein and DL-methionine as the sole source of dietary amino acids. The effect of such a regime was evaluated by observations on postweaning weight gains, on the reproductive performance and on the incidence of "acute uremia of the newborn"(20) among the litters produced.

**Experimental.** The basal ration ( $S_2$ ) used has been previously described(19). It consisted of a commercial soybean protein (Archer-Daniels-Midland Co.), DL-methionine, sucrose, hydrogenated vegetable oil, corn oil, salts and suitable supplements of all available vitamins except ascorbic acid and vitamin  $B_{12}$ . In the ration of half of the animals 2% of PST (Sharp and Dohme, veterinary sulfathalidine) replaced an equal quantity of sucrose. The rations were fed *ad libitum*. The rats used for these experiments were  $F_2$  or  $F_3$  generation offspring from mothers maintained continuously on the basal ration. Carefully matched littermate females were divided at 21 days of age into 2 groups and fed the rations referred to above. The rats were housed in groups of 3-4 animals in cages with raised wire screens. When the females were 10 weeks old they

were bred with males maintained on the same rations. Shortly before parturition and during lactation the females were transferred to individual cages containing clean wood shavings. After weaning or loss of a litter the rats were returned immediately to the breeding cages. The experiment was terminated when most of the females on the PST ration had weaned 2 litters.

**Results and discussion.** The data summarized in Table I suggest that the addition of 2% of phthalylsulfathiazole to the ration of these rats supported better performance by every criterion applied. However, the differences in favor of the ration containing the bacteriostatic compound are statistically significant ( $P < 0.05$ ) only with respect to the incidence of acute uremia of the newborn, the 21 days weight of the young and the weight change of the mothers during lactation.

The most striking effect of the addition of PST to the basal ration used in this experiment was the elimination of the syndrome of acute uremia of the newborn. This condition was encountered in the group consuming the basal ration with about the same incidence as in other, more extensive experiments(21). While the etiology of acute uremia of the newborn is not clear the syndrome can be

20. Schultze, M. O., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 613.

21. Halvorson, H. O. and Schultze, M. O., *J. Nutr.*, 1950, in press.

largely prevented by the feeding of materials known to be high in vit. B<sub>12</sub> activity(21) to the mothers or by injection of vit. B<sub>12</sub> into the young(20). The results presented in Table I could therefore be interpreted as indicating that in the presence of PST the intestinal flora was altered so that microbiological synthesis of vit. B<sub>12</sub> provided the maternal organism with a supply of this compound sufficient to prevent the appearance of the external symptoms of acute uremia in the newborn rats. Since absolutely specific methods for the determination of vit. B<sub>12</sub> are not available at present a decision as to whether microbiological synthesis of vit. B<sub>12</sub> was in fact responsible for the beneficial effects of PST reported here must be held in abeyance. The components of a ration may have a marked effect on gastrointestinal synthesis of vitamins(22). Thus, it has been reported(23) that the feces of rats maintained on an all plant ration were persistently negative for vit. B<sub>12</sub> activity while those pro-

22. Wright, L. D., Skeggs, H. R. and Sprague, K. L., *J. Nutr.*, 1945, v29, 430.

23. Zucker, T. F. and Zucker, L. M., Abstracts 117th Meeting Am. Chem. Soc., 1950, p. 16A.

duced on a casein ration contained vit. B<sub>12</sub> activity. In this connection it might be pointed out that the ceca of the rats consuming PST were in this experiment always found to be greatly enlarged compared to those of animals consuming the basal ration. This was observed even in 21-day-old weanling rats which had consumed the ration for only a few days. Baxter(17) has recorded a similar observation.

*Summary.* The feeding of 2% of phthalyl-sulfathiazole to rats maintained on a purified ration containing a commercial soybean protein and DL-methionine as the only source of amino acids produced no depression of growth; it supported better reproductive performance and no cases of acute uremia of the newborn were observed among about 200 rats. On the basal ration, acute uremia of the newborn occurred in 35% of the litters born.

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### Effect of ACTH on Glycogenesis and Glycolysis in Hypophysectomized Rats. (18098)

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Abelin(1) showed that after feeding starved rats a high carbohydrate diet, an increase in liver glycogen accompanied a 25% reduction in adrenal cholesterol. He suggested that the rise in blood sugar may have stimulated the pituitary to secrete ACTH, which caused a release of adrenocortical hormone, and this, in turn, stimulated liver glycogenesis. Ingle(2) was able to demonstrate

adrenal hypertrophy in rats which died as a result of an excessive carbohydrate diet. Similar results were reported by other workers (3,4). Hypophysectomy caused a decrease in the muscle glycogen of rats, but the glycogen level was restored to normal upon the administration of anterior pituitary extract; however, pure ACTH was without effect(5)-

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3. Foglia, V. G., *Rev. soc. Argent. biol.*, 1945, v21, 45.

4. Bennett, L. L., and Koneff, A. A., *Anat. Rec.*, 1946, v96, 1.

\* Present address: Department of Physiology, University of Chicago.

1. Abelin, I., *Schweiz. med. Wochsh.*, 1946, v76, 527.

## ACTH ON GLYCOGENESIS AND GLYCOLYSIS

Liver glycogen dropped slightly in hypophysectomized animals but was not increased by administering anterior pituitary extract or ACTH. Previously Russell and Craig(6) demonstrated that adrenocortical extracts increased liver glycogen.

The present study was undertaken in order to investigate the glycogenesis and glycolysis in the hypophysectomized rat and, then, to determine the effect of ACTH. An analysis of phosphorylated intermediates as well as acid-insoluble phosphorus components was conducted in the hope of gaining some insight into the mechanisms involved in energy turnover.

**Methods.** Sprague-Dawley rats were hypophysectomized when 40 days old and used 12 days later. The diet was Purina Dog Chow. Four hypophysectomized rats received 10 mg of a long-acting preparation of ACTH 24 hours before sacrifice. All animals received 2 cc of a saturated solution of glucose by stomach tube 3 hours before sacrifice.

Analyses for the phosphorylated intermediates were conducted according to the method outlined by LePage and Umbreit(7). The acid-insoluble phosphorus was determined by the method of Schneider(8). Aerobic glycolysis was measured by the method of LePage(9).

**Results.** Hypophysectomized rats showed a considerably decreased ability to synthesize liver glycogen from glucose, the inhibition being greater than 50% (Table I). In hypophysectomized rats treated with ACTH the liver glycogen content was essentially normal. Likewise, the glycogen level of the brain which was reduced in hypophyse-

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6. Russell, J. A., and Craig, J. M., *Proc. Soc. EXP. BIOL. AND MED.*, 1938, v39, 59.

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8. Schneider, W. C., *J. Biol. Chem.*, 1945, v161, 293.

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TABLE I. Phosphorylated Intermediates of the Liver of Normal and Hypophysectomized Rats With and Without ACTH.

The values are in  $\mu\text{g}/\text{wt wt}$  and represent an average of 2 rats, the determinations agreeing within 20%.

	Normal	Hypophysec-tomized	Hypophysec-tomized ACTH
Glycogen	48,000	18,000	44,000
Glucose-1-phosphate	120	160	140
Glucose-6-phosphate	1,380	1,330	2,000
Fructose-6-phosphate	170	180	150
Fructose-1,6-phosphate	25	28	138
Triose phosphorus	77	22	20
ATP	120	900	125
ADP	670	84	690
Adenylic acid	700	440	880
Total phosphorus	920	1,200	670
Inorganic phosphorus	240	470	265

TABLE II. Phosphorylated Intermediates of Brain of Normal and Hypophysectomized Rats With and Without ACTH.

Values are in  $\mu\text{g/g}$  wet wt and represent an average of 2 rats, the determinations agreeing within 20%.

	Normal	Hypophysec-tomized	Hypophysec-tomized ACTH
Glycogen	500	320	690
Glucose-1-phosphate	200	210	42
Glucose-6-phosphate	1,400	1,600	1,500
Fructose-6-phosphate	38	40	164
Fructose-1,6-phosphate	40	42	240
Triose phosphorus	10	10	17
ATP	600	450	590
ADP	10	110	236
Adenylic acid	580	480	465
Phosphocreatine	610	520	800
Total phosphorus	910	915	960
Inorganic phosphorus	320	470	425

mized rats was restored to normal by the administration of ACTH (Table II). The ATP content of the liver of normal and hypophysectomized animals treated with ACTH was considerably less than in the untreated hypophysectomized rats; whereas the reverse was true for ADP. In the former the ratio of ATP to ADP was 1 to 7, while in the latter the ratio was 10 to 1. Adenylic acid was about 50% lower in hypophysectomized animals than in the normals or ACTH-treated. In the brain the ATP con-

TABLE III. Acid-insoluble Phosphorus Components of Liver and Brain on Normal and Hypophysectomized Rats With and Without ACTH.  
Values are in  $\mu\text{g/g}$  wet wt and represent an average of 2 rats, the determinations agreeing within 30%.

	Liver			Brain		
	Normal	Hypophysec.	Hypophysec. ACTH	Normal	Hypophysec.	Hypophysec. ACTH
Phospholipid P	1,170	1,500	1,250	1,700	1,850	1,800
Nucleic acid P	450	450	390	300	270	250
Phosphoprotein P	530	300	460	550	260	530

tent of the hypophysectomized rats was somewhat lower than in the other animals. The fructose-1,6-phosphate was significantly greater in the liver and brain of hypophysectomized rats given ACTH. Total and inorganic phosphorus were higher in the hypophysectomized rats than in the normals or hypophysectomized rats receiving ACTH.

The phospholipid and nucleic acid phosphorus content of hypophysectomized rats showed no significant variation from the normals (Table III). Phosphoprotein phosphorus, however, decreased up to 50% in the hypophysectomized rats as compared to the normals and hypophysectomized rats treated with ACTH.

A typical experiment of the anaerobic glycolysis of the brain of normal and hypophysectomized rats with and without ACTH is represented in Fig. 1. It can be seen that the rate of glycolysis of hypophysectomized rats is over 20% less than the normal or hypophysectomized rats given ACTH.

*Discussion.* The relative inability of hypophysectomized rats to synthesize liver glycogen is in accord with the results of Abelin(1). That the adrenal cortex is specifically involved here is evident from the fact that ACTH restores the glycogenetic capacity of the liver of hypophysectomized rats to normal. It is interesting in this respect that liver ATP is converted to ADP during glycogen synthesis, and that when glycogen synthesis is impaired, as in the hypophysectomized animals, the ratio of ATP to ADP is high. Glycogenesis must be a form of stress in the liver enlisting high energy phosphate bonds in order to continue. It is not clear from these results whether or not the high ratio of ATP to ADP is antecedent to the

reduced glycogenetic capacity of the liver of hypophysectomized rats; however, the decreased rate of glycolysis in the brain of hypophysectomized rats is suggestive of some interference with energy turnover. The high inorganic phosphorus and low adenyllic acid content of untreated hypophysectomized animals are indicative of an interference with phosphorus utilization. Preliminary studies have revealed no inhibition of pyruvate, succinate, and glutamate oxidation in tissues of untreated hypophysectomized rats.

*Summary.* An analysis of the phosphorylated intermediates of the liver and brain of hypophysectomized rats with and without ACTH was made. The decreased ability of hypophysectomized rats to synthesize glycogen from glucose was restored to normal by ACTH. Phosphoprotein phosphorus levels also returned to normal with ACTH. The

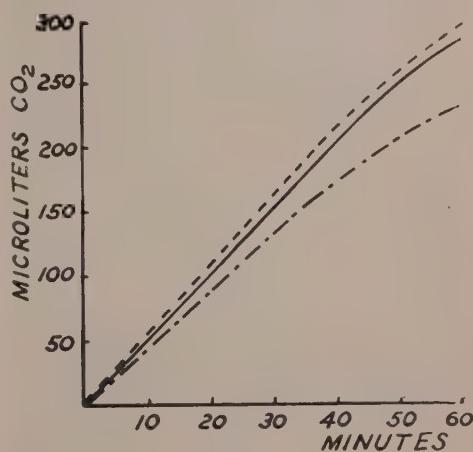


FIG. 1.  
Anaerobic glycolysis of brain of hypophysectomized rat given ACTH (---), of normal (—), and of untreated hypophysectomized rat (·—·).

anaerobic glycolysis of the brain of hypophysectomized rats was reduced 20% below that of normal rats but this was restored to normal

by the administration of ACTH.

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### Adrenal Medullary Hormones in Water Diuresis.\* (18099)

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Recent studies have established the existence of nor-epinephrine both in the adrenal medulla and in natural U. S. P. epinephrine and it is now generally believed that this substance is normally secreted by the adrenal glands(1-4). Many workers(e.g. 5-8) have found nor-epinephrine to have biological and pharmacological properties different from those of epinephrine and it has become of general interest and importance to determine which of the many actions ascribed to adrenal medullary secretions are due to epinephrine, nor-epinephrine or both. The diuretic response to water offers an easy and convenient testing procedure for the investigation of one possible difference in the action of these compounds since it has been established that large doses of adrenalin (nor-epinephrine and epinephrine) greatly stimulate the flow of urine in water diuresis(9). The purpose of

the experiments reported here, therefore, was to determine whether one or both of the adrenal medullary hormones was responsible for the augmentation of urine flow such as that obtained by Gaunt *et al.*(9). The results indicate that nor-epinephrine rather than epinephrine was the causative agent involved.

*Methods.* Four types of adrenal medullary hormone preparations<sup>†</sup> were employed. The adrenalin (Parke-Davis) was predominately l-epinephrine but contained small amounts of nor-epinephrine. The pure compounds used were dl-nor-epinephrine hydrochloride, l-epinephrine bitartrate and l-nor-epinephrine bitartrate monohydrate. Each of these was suspended in peanut oil and administered so that the volume of oil was kept constant (0.05 ml per 100 g body weight) although the amount of hormone varied from 3 to 30 µg per 100 g body weight. Female rats, weighing approximately 200 g were fasted 18 hours but allowed water *ad libitum*. They were placed in individual metabolism cages, injected subcutaneously with hormone, and given orally one dose of water (3 ml per 100 sq cm of body surface)(11) warmed to body temperature. Urine output was recorded at half-hour intervals for a period of 3 hours.

\* This investigation was supported by a research grant from the National Heart Institute, U. S. Public Health Service.

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† We are indebted to Dr. F. F. Yonkman of the Ciba Pharmaceutical Products, Inc., for the dl-nor-epinephrine, to Mr. B. F. Tullar of the Sterling-Winthrop Research Institute for l-epinephrine bitartrate and l-nor-epinephrine bitartrate monohydrate, and to Dr. M. L. Moore of Smith, Kline and French Laboratories for Dibenamine hydrochloride.

TABLE I. Showing Mean Effect on Water Diuresis Produced by Various Types of Adrenal Medullary Hormones in Intact Rats.

Series	Type of preparation	No. of animals	% water excreted at indicated min.	
			30	60 ± S.E.*
1	Controls	19	3.3	30.5 ± 3.7
2	Adrenalin, 30 γ	11	9.5	43.8 ± 5.9
3	DL-nor-epinephrine, 30 γ	12	29.3	79.7 ± 7.6
4	L-nor-epinephrine, 30 γ	12	26.2	84.5 ± 8.2
5	L-epinephrine, 30 γ	12	5.2	34.1 ± 5.1
6	Adrenalin, 3 γ	12	15.1	45.6 ± 5.8
7	DL-nor-epinephrine, 3 γ	11	31.3	61.0 ± 6.2
8	L-nor-epinephrine, 3 γ	14	8.8	49.9 ± 6.9
9	L-epinephrine, 3 γ	15	6.0	27.9 ± 4.9

$$S.E. = \pm \sqrt{\frac{\sum d^2}{n(n-1)}}$$

The rate of excretion of the administered water was the major criterion of response. Control rats were injected subcutaneously with 0.05 ml peanut oil per 100 g body weight, but otherwise they were handled in the same manner as those receiving hormones.

Since N, N-dibenzyl-beta-chlorethylamine (Dibenamine) is known to block the action of administered epinephrine(12), it was decided to use this drug in a series of experiments. A 5% solution of commercially prepared dibenamine hydrochloride was diluted with 0.9% saline so that 1 ml contained 10 mg. This was injected subcutaneously in the amount of 0.5 mg (0.05 ml) per 100 g body weight, 14 hours prior to water or hormone administration.

In order to study the effects of hormones and dibenamine in animals without adrenal medullary secretion another group of animals were medullectomized 3 to 4 weeks prior to testing in order to allow regeneration of the cortex. The operations were carried out under ether anesthesia and the glands enucleated by pressure after their capsules had been nicked.

**Results.** As seen in Table I, the injection of adrenalin was less effective in stimulating diuresis than was nor-epinephrine. Estima-

tions taken from the increment in urine flow over the control value at the 60 minute interval indicated that 30 µg of dl-nor-epinephrine was about 3 times more effective than the same quantity of adrenalin, and at the 3 µg level the dl-nor-epinephrine was 2 times more effective than adrenalin. Such results pointed toward the possibility that the diuretic action of the adrenalin used was due to its nor-epinephrine content; this was further borne out by the fact that l-epinephrine failed to augment urine flow whereas l-nor-epinephrine was highly effective in this respect.

Although it is not shown in the tables, the diuretic response obtained with adrenalin and the nor-epinephrine compounds lasted about 2 hours. At the end of 3 hours, the controls had excreted over 80% of the administered water and had approached, but not equalled, the excretion level of those animals that had been treated.

Obvious signs of overdosage, such as gasping, stretching, twisting, etc., lasted for about 90 minutes after the injection of the higher doses of all preparations. At the 3 µg level, however, dl-nor-epinephrine produced no grossly observable overdosage effects, while l-nor-epinephrine produced detectable responses; adrenalin and l-epinephrine elicited marked but less severe reactions of the types noted above with larger doses. We do not know whether this low dosage is within a physiological range because estimations on the rate of secretion of nor-epinephrine have

11. Benedict, F. G., Vital Energetics, Carnegie Institution of Washington, Washington, D. C., 1938, p. 174.

12. Nickerson, M., *J. Pharmacol. and Exp. Therap.*, 1949, v95, 27.

TABLE II. Showing Mean Effect on Water Diuresis Produced by Adrenalin and DL-nor-epinephrine in Rats Treated with Dibenamine.

Series	Type of preparation	No. of animals	% water excreted at indicated min.	
			30	60 ± S.E.
1	Controls	19	3.3	30.5 ± 3.7
2	Dibenamine, .5 mg	11	1.5	21.4 ± 4.7
3	Dibenamine, .5 mg + adrenalin, 30 γ	12	1.4	5.5
4	Dibenamine, .5 mg + DL-nor-epinephrine, 30 γ	11	4.4	43.5 ± 5.2

TABLE III. Showing Mean Effect on Water Diuresis Produced by DL-nor-epinephrine and Dibenamine in Adrenal Medullectomized Rats.

Series	Treatment	No. of animals	% water excreted at indicated min.	
			30	60
1	Medullectomized	11	9.0	33.7 ± 3.9
2	Medullectomized + DL-nor-epinephrine, 3 γ	11	25.8	68.9 ± 4.3
3	Medullectomized + .5 mg dibenamine	11	18.5	40.8 ± 4.3
4	Medullectomized + .5 mg dibenamine + DL-nor-epinephrine, 30 γ	9	24.1	76.4 ± 6.6

not as yet been made.

*Effects of treatment with dibenamine.* As seen in Table II, a small amount (0.5 mg) of dibenamine caused a reduction in urine flow. Higher doses of this drug were used but the results are not tabulated here; 2½ mg per 100 g of body weight, the dosage employed by Tepperman and Bogardus(13) in other experiments, was antidiuretic to such an extent that urine flow was almost completely inhibited for 2 hours. The dibenamine blocked and reversed the otherwise diuretic action of adrenalin but only partially blocked the diuresis caused by nor-epinephrine.

*Effects of demedullation (Table III).* In confirmation of Gaunt *et al.*(9) animals lacking an adrenal medulla responded to diuresis tests in a normal fashion, and when such animals were treated with 3 µg of nor-epinephrine they responded as well as did intact controls. The injection of dibenamine into medullectomized rats did not cause a reduction in the diuretic response to water as it did in intact animals. Furthermore, when 30 µg of DL-nor-epinephrine was administered to demedullated dibenamized rats the nor-epinephrine was just as effective as

when given to intact controls. In other words, under these conditions, dibenamine did not alter the diuretic effect of nor-epinephrine.

*Discussion.* The results reported here show clearly that nor-epinephrine exhibits a diuretic influence when injected subcutaneously in intact rats which is approximately three times greater than that seen with adrenalin. That the diuretic action of the adrenalin used is due to its nor-epinephrine content seems plausible in view of the fact that pure L-epinephrine failed to augment urine flow. Such findings may be of help in reconciling conflicting reports in the literature concerning urine flow after the administration of adrenal medullary hormone preparations(9, 10, 14, 15) although the mode of administration and the species employed may have to be taken into consideration in arriving at any generalized conclusions. The finding that adrenal demedullated animals respond to a water diuresis test in no way different from intact animals confirms the work of Gaunt *et al.*(9) but is at variance with the results of Stein and Wertheimer(10). The basis for the discrepancy between these two

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groups of workers is still obscure. The problem of the part played by the adrenal medulla in water metabolism under normal conditions is one of considerable importance and the finding reported here that small doses of dl-nor-epinephrine stimulate water diuresis without giving other gross overdosage effects makes it feasible to seek further for evidence of a physiological role for the adrenal medulla in such processes. We intend to explore such possibilities in extension of this work by attempting an analysis of these actions of nor-epinephrine in terms of circulatory and renal mechanisms. It is pertinent to point out that the lack of gross overdosage effects with dl-nor-epinephrine may be due to the lesser toxicity of nor-epinephrine as compared with epinephrine(8).

It is well established that dibenamine will block and often reverse some of the actions of epinephrine(12). This proved to be the case in our experiments for adrenalin but a reversal effect was not seen with nor-epinephrine. In other words, in the intact rat, dibenamine more effectively blocks the action of injected adrenalin than it does nor-epinephrine. However, in the absence of the

adrenal medulla dibenamine does not have any blocking action on nor-epinephrine and its characteristic water retaining properties are absent. It is possible that the blocking action of dibenamine on water diuresis is dependent upon the presence of either endogenous or administered medullary epinephrine. It would be of interest to see if this holds true for other types of dibenamine blockage.

*Summary.* The injection of non-epinephrine in the rat is more effective in causing an augmentation of water diuresis than is adrenalin. Pure l-epinephrine has no stimulating action on diuresis.

Dibenamine causes a water retention in intact rats but fails to do so in adrenal medullectomized rats.

Dibenamine blocks and reverses the diuretic activity of adrenalin in intact rats but it less effectively blocks such activity of nor-epinephrine. Dibenamine, in the dosage used, was incapable of blocking the action of nor-epinephrine in medullectomized animals.

These results indicate that the diuretic action of adrenal medullary hormone is not due to epinephrine but to the presence of nor-epinephrine.

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### Influence of Blood Incompatibilities on Measurement of Blood Volume by Cell-Tagging Methods.\* (18100)

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During a recent series of direct comparisons in dogs of blood volume determined with radio iron ( $Fe^{55}$ ), CO and dye (T-1824)(1),

we sometimes encountered reactions to the injections of donor blood. In one dog the reactions were so severe that the tests were discontinued. In a second animal with very severe reactions we decided to complete the test and then found that both the CO volume, which normally agrees with the dye-volume, and the radio-iron volume were extremely low as compared with the results of the dye determination. Subsequently, we noted that if the blood for CO saturation and injection was drawn from the recipient dog itself in-

\* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service, in part by the Atomic Energy Commission and also in part by a grant from the Baruch Fund for Physical Medicine.

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stead of from the donor, reactions to the injection occurred less frequently and the agreement in volume measured with CO and dye was more consistent. Yet the ratio of blood volume measured by radio iron and by dye still revealed large fluctuations (0.7 to 1.0). These and other observations led us to investigate the effect of blood incompatibilities on the blood volume measurements.

The cross-matching was performed according to the procedures developed by Dr. Angie Hamilton of Philadelphia(2). In tests on the dogs still available from earlier experiments it was found that the experiments in which the iron values were low relative to dye or CO values were those in which the iron donor cells showed clumping reactions in the recipient dog's serum.

In order to explore this matter further the blood volume of the same dog was measured in successive tests with matching and non-matching donor bloods. Where matching was satisfactory and the dog showed no overt reactions to the donor bloods, the values from the different methods agree fairly well. Where either the radio iron or CO donor cells showed incompatibility in the recipient's serum, the recipient had definite reactions (one animal died in shock 3 hours after the injections) and the radio-iron volume and/or CO volume were low compared with the dye-volume. However in these repeated tests the total blood volume by the dye method was nearly the same whether or not reactions were present, thus indicating that the iron and CO measurements were the ones most affected by the untoward reactions.

These observations suggest that when the cells are clumped in the reactions of incompatibility the tagged cells may not be distributed evenly throughout the circulation, especially in the small vessels, and that under these circumstances a cell-tagging method may

underestimate the volume.

The literature fails to reveal that any attention has been paid to the question of blood matching in blood volume determinations in dogs. In experiments on man with radio iron or with the Ashby marked cell method, universal donors are used. Yet careful observers report the occurrence of occasional reactions(3). The P<sup>32</sup> method(4,5), seemingly eliminates the problem of blood incompatibilities. However, a few preliminary tests have given indication that dog blood after incubation with P<sup>32</sup>, and occasionally after saturation with CO, shows slowly appearing clumping reactions with fresh serum from the same animal.

The possible effect of these factors on the measurement of blood volume with various cell-tagging methods are being studied in more detail.

**Summary.** A study of the blood volume of the dog as measured simultaneously by the Dye T-1824, by carbon monoxide and by radioactive iron, Fe<sup>55</sup>, has shown that the values measured by the cell-tagging methods are distinctly lower than the value given by the dye method whenever the donor blood tagged with CO or Fe<sup>55</sup> shows incompatibility with the blood of the recipient animal. However, when the bloods of the donor and the recipient animals show no incompatibility, the results by the three methods show improved agreement. These results suggest that the cell-clumpings occurring in the reaction of incompatibility, by making the smaller vessels inaccessible to the clumped cells, reduce the volume measured by the tagged cells.

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## Binding of the Mercury of an Organic Mercurial Diuretic by Plasma Proteins.\* (18101)

J. PERVIS MILNOR† (Introduced by G. E. Burch)

During investigations of the time-course of various elements in the body, certain differences were noted between the behavior of  $\text{Na}^{22}$  and  $\text{Na}^{24}$ , introduced as a mercurial diuretic (Mercurydrin‡) (1-5). With comparable concentrations in the serum, there were differences in both time of appearance and concentration of the isotopes in various fluids of the body (1,2,4). These physiologic data suggested a tendency for the radiomercury to bind to plasma proteins, thus influencing the transfer of the isotope around the body. To investigate the possible relationship of protein binding of the radiomercury upon its transfer across vascular membranes *in vivo*, observations were made on the diffusion of the mercury of the mercurial diuretic across a semipermeable membrane *in vitro*.

*Materials and methods.* Test solutions containing the labeled mercurial diuretic con-

sisted of human Ringer's solution, reconstituted dried human plasma, fresh human plasma and human urine. Dialyzing bags were made of cellophane sausage casing, impermeable to plasma proteins. All dialyses were performed in duplicate. Samples of test solutions and bathing solutions were taken before and after dialysis and were prepared and counted according to methods previously described (6,7). Two minims of a solution containing 50,000 units of penicillin per cc was added to each bag and bath to prevent bacterial growth. The reconstituted plasma was found to have a total protein of 7.3 g per 100 cc and albumin of 4.9 g per 100 cc.

*Results.* Results of many experiments are summarized in Tables I and II. The dialysis of Mercurydrin in Ringer's solution (Table I, Exp. 2) showed a decrease in transfer of mercury across the membrane. This suggests that the mercury was bound to the plasma proteins. From the dialysis of Mercurydrin in Ringer's solution placed inside the cellophane bag against plasma as the bath (Table I, Exp. 3) it is apparent that the plasma proteins bound the mercury which diffused into it. At equilibrium, the mercury in the Ringer's solution was "free" and was probably in equilibrium with a similar concentration of free mercury in the bag. The concentration of "bound" mercury in the plasma is equal to the total concentration of mercury minus the concentration of "free" mercury. Thus, the mercury was respectively 96% and 94% protein-bound (Table I, Exp. B and C).

When relatively large concentrations of mercury were present, the mercury diffused across the membrane in quantity despite the

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† National Institutes of Health Postdoctorate Research Fellow (1948-50), Department of Medicine, Tulane University School of Medicine and Charity Hospital of Louisiana at New Orleans.

‡ The sodium salt of methoxy-oximercuripropylsuccinylurea with theophylline, prepared with radiomercury in this laboratory by Messrs. Harold Krahne, Darwin Kaestner and Edwin Sprengler through the courtesy of Dr. H. L. Daiell, Director of Research, Lakeside Laboratories, Milwaukee.

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## MERCURIAL BINDING BY PLASMA PROTEINS

TABLE I. Dialysis of 10 cc Volumes of 1:2000 Dilution of Mercuhydrin for 120 Hr at 7°C.

Exp. No.	Solution in		Conc. of radiomercury in		Mercury in plasma	
	Dialyzing bag	Bath	Bag, CPM/cc	Bath, CPM/cc	Bound, %	Free, %
1	Ringer's	30 cc vol. Ringer's	1177	1173		
2	Plasma	Ringer's	4213	183	96	4
3	Ringer's	Plasma	96	1690	94	6

CPM = Counts per minute.

TABLE II. Dialysis of 10 cc Volumes of Various Dilutions of Mercuhydrin in Plasma in Dialyzing Bags.

Exp. No.	Solution in		Initial dilution of mercuhydrin	Final conc. Hg, μg/cc	Conc. of sol. in		Mercury in plasma	
	Dialyzing, bag	Bath, Ringer's			Bag, CPM/cc	Bath, CPM/cc	Bound, %	Free, %
4a*	Plasma	30 cc	1:1000	28.9	8566	340	96	4
b	"	"	1:500	52.7	15623	1813	88	12
c	"	"	1:100	162.5	48766	18926	61	39
5a†	"	10 cc	1:800	45.9	238	22	92	8
b	"	"	1:400	78.0	468	94	80	20
c	"	"	1:200	144.4	836	314	63	37
d	"	"	1:100	260.0	1487	612	45	55
e	"	"	1:50	487.5	2750	1916	30	70

\* 120 hr, 70°C.

† 5 hr, 37°C, with constant agitation.

presence of plasma (Table II, Exp. 4c, 5c, d and e). Thus, the quantity of mercury bound by the plasma appears to be a function of mercurial concentration.

Other similar experiments indicated that the mercury of the mercurial diuretic was similarly bound by the plasma proteins after intravenous administration of the drug. The mercury in normal and protein-free urine, on the other hand, remained dialyzable.

**Discussion.** Until comparatively recently, observations regarding mercury-protein interactions have been concerned with denaturative phenomena obtained with relatively high concentrations of mercury(8). Hughes(9,10), by ultracentrifugal and chemical methods, noted soluble mercury-albumin complexes consisting of one molecule of mercury to one or two of albumin. On the basis of these studies he postulated a single reactive sulf-

hydryl group per molecule of the albumin by which the mercury is bound(10). Other reactive sulphydryl groups are to be found among the globulins(11), and it is probable that mercury is bound by them as well. Studies utilizing the ultracentrifuge have not shown measurable "free" mercury either in a 1:2 molar mixture of a mercuric compound with human serum albumin *in vitro*(11) or in plasma obtained from a human subject 10 to 30 minutes after intravenous injection of the mercurial diuretic(12). The consistent presence of mercury outside the bags in these experiments (Table II) indicates that under these conditions there is some "free" mercury even in relatively dilute solutions.

These observations have many interesting biologic implications. For example, studies on renal extraction and excretion of mercury (5) revealed that 25 to 50% of the mercury could not be accounted for in the circulating blood or in the kidney within 5 minutes after injection of the mercurial diuretic. This loss of mercury cannot be explained by dif-

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fusion out of the vascular system of mercury attached to proteins(13) nor can the disappearance of the mercury be accounted for in extracellular fluid obtained from various sites(4). Within the first minute or so after injection, mercurial dilutions of 1:500 and less exist(1). At these dilutions, there should be appreciable quantities of nonprotein bound mercury. It would appear, therefore, that much of this initially "free" mercury becomes bound to structures of or immediately adjacent to these blood vessels.

*Summary and conclusion.* The experiments herein reported corroborate the observations

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of other investigators that the mercury of an organic mercurial diuretic may be bound by the proteins of the plasma. The extent of this binding is a function of mercurial concentration; at dilutions of the diuretic of 1:1000 or higher the mercury is over 90% bound; at dilutions of 1:100 or lower, the mercury is over 50% free. This may well explain certain phenomena observed in the tracer studies of the mercurial diuretics.

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### Diurnal Variation in the Plasma Iron Level of Man.\* (18102)

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During a study of factors affecting the level of iron in the plasma of man, it became apparent that the plasma iron level normally undergoes a regular diurnal variation. Reference to the literature shows that several continental investigators have described such a variation. Anglo-American workers have either failed to observe this, taken their samples at a constant time, or ignored the variation in their studies on plasma iron.

*Methods.* In this study 19 adult male subjects, without demonstrable organic disease which would affect the plasma iron, were used. Samples of approximately 8.0 ml of blood were taken at intervals of 2 to 4 hours throughout a 24-hour period from 12 subjects beginning at 9:00 a. m. and from 7 subjects beginning at 5:00 p. m. Glassware used for the collection, storage and estimation of plasma iron was carefully cleansed by first rinsing with water and then immersing in

potassium dichromate and sulfuric acid cleaning solution for a minimum of one hour. It was then rinsed thoroughly with tap water and this was followed by three washings in double distilled water prepared in an all glass distillation apparatus. The blood was taken with all glass syringes and placed into 15 ml tubes to which 2 drops of 20% potassium oxalate had been added previously. The plasma was then separated by centrifugation and the iron estimation made by a modification of the method of Barkan and Walker(1).

*Reagents.* 1. Hydrochloric acid, 1.2% solution in double distilled water. 2. Trichloroacetic acid (redistilled), 20% solution in double distilled water. 3. Thioglycolic acid (Eastman Kodak-Practical). 4. O-phenanthroline monohydrate, 0.1% solution in double distilled water. 5. Sodium acetate, saturated solution in double distilled water.

*Procedure.* To 1.5 ml of plasma or serum, 0.75 ml of 1.2% hydrochloric acid is added. The plasma is then incubated at 37°C for

\* This study was aided by a grant from the United States Public Health Service.

† Postdoctorate Research Fellow of the United States Public Health Service, 1949-1950.

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1 hour, after which time 0.75 ml of 20% trichloroacetic acid is added, the resulting precipitate being stirred vigorously. The mixture is then allowed to stand at room temperature for 1 hour. The supernatant is separated by centrifugation and an aliquot of 1.80 ml is taken. To this are added in the following order, with mixing after each addition, 0.05 ml of thioglycolic acid (2 drops), 0.4 ml of 0.1% O-phenanthroline, 0.4 ml of saturated sodium acetate solution and 0.35 ml of water, making a total of 3.0 ml. A blank is prepared by adding the same reagents to 0.45 ml hydrochloric acid, 0.45 ml trichloroacetic acid and 1.25 ml water. The test and blank solutions are then allowed to stand at room temperature for 30 minutes, which is adequate for the full color reaction of the ferrous iron with O-phenanthroline to develop. In this modification of the Barkan and Walker method, thioglycolic acid has been found to be a more satisfactory reducing agent than hydrazine sulfate. The addition of the O-phenanthroline before the sodium acetate facilitates the more rapid development of the color reaction. Absorption is measured in the Beckman spectrophotometer at 510  $\mu\text{m}$  with a slit width of 0.032 mm. Under these conditions, using cuvettes with a light path

Concentration  
of 1.0 cm, a constant K ( $K = \frac{\text{absorbance}}{\text{concentration}}$ )  
Density

of 14.87 was obtained using standard iron solutions where the concentration was expressed in micrograms of iron per 3.0 ml.

**Results.** From the figure it can be seen that the level of plasma iron is highest in the early morning and that it falls during the day to its lowest level in the evening, and rises again during sleep.

Blood samples were taken throughout the 24 hour period at intervals beginning at 5:00 p.m. since it might be argued that the diurnal variation observed above was the result of repeated vena-puncturing and sampling. The mean plasma iron level at 5:00 p.m. was  $66 \pm 14 \mu\text{g} \%$  and this rose during the night, while the samples were being taken, to an average of  $148 \pm 11 \mu\text{g} \%$  in the early morning, a difference of  $82 \pm 15 \mu\text{g} \%$ . This average maximal value compares well with

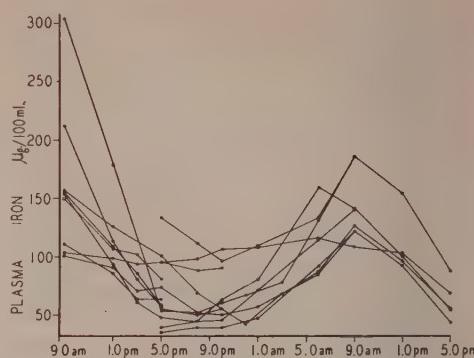


FIG. 1.  
The diurnal variation in the plasma iron of 19 individuals.

the average early morning values found in the 12 subjects on whom bleeding began at 9:00 a.m. The declines in plasma levels from morning to evening, it will be noted, were also similar. Thus the variation is not related to the taking of blood samples.

**Discussion.** The above observations indicate that the plasma iron of man undergoes a definite diurnal variation. These observations are in agreement with the results of others(2-7) which have been summarized, analyzed statistically, and presented in Table I. In addition to the tabulated studies, those of Nilsson may be mentioned. He was reported by Höyer(3) to have published a 24 hour curve for serum iron in a normal subject which showed a decrease at night, followed by an increase the next morning. Schäfer(8) also noted a similar diurnal variation in 10 healthy subjects with a mean decrease of  $22 \mu\text{g} \%$ . It can be seen that the diurnal variation was greater in our subjects and in those reported in Höyer's second paper(4) than is given by the other workers(2,3,5,6,7).

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TABLE I. Diurnal Variations in the Plasma Iron of Normal Human Subjects.

Investigator	No. and sex of subjects	Plasma iron morning, $\mu\text{g}\%$	Plasma iron evening, $\mu\text{g}\%$	Difference, $\mu\text{g}\%$
This investigation	12 males	155 $\pm$ 16	65 $\pm$ 5	89 $\pm$ 18
Hemmeler, '44	25 "	127 $\pm$ 6	82 $\pm$ 6	45 $\pm$ 7
" "	23 "	106 $\pm$ 6	74 $\pm$ 4	32 $\pm$ 7
Hoyer, '44, till 11 p.m.	6 "	129 $\pm$ 11	88 $\pm$ 7	41 $\pm$ 6
" " " "	6 females	114 $\pm$ 4	70 $\pm$ 4	44 $\pm$ 8
" " through night	9 males	131 $\pm$ 11	76 $\pm$ 6	56 $\pm$ 9
" " " "	11 females	130 $\pm$ 10	66 $\pm$ 5	64 $\pm$ 11
Valquist, '41	15 "	135 $\pm$ 11	99	36 $\pm$ 9
Waldenström, '46	15 "	113 $\pm$ 8	79 $\pm$ 4	34 $\pm$ 9
Heilmeyer, '37	1 male	93	72	21

This may be due to the fact that in our study, as in Höyer's second investigation, samples were taken at intervals throughout a whole 24 hour period and in this way lower levels of plasma iron may have been found.

Moore, Minnich and Welch(9) failed to find such a variation. These workers took 6 or 7 blood samples at intervals throughout the day from an unspecified number of normal subjects and hospital patients. The fluctuations they found were not constant in direction. Wetzel is quoted by Hemmeler(2) as having found in a series of 11 subjects that the plasma iron increases during the day. These appear to be the only conflicting results in the literature although it may be noted that most workers report some variations from the general trend in a few individuals in their series. Thus, of Valquist's 15 men, 2 showed an increase of plasma iron level in the evening sample, and, in our series of 12 subjects, there was one whose iron level showed a decrease of only 10  $\mu\text{g}/100 \text{ ml}$  during the day.

The diurnal variation in the plasma iron would seem to be independent of the initial plasma iron level. Thus Hemmeler(10) found that in 16 cases of infectious hepatitis, a disease in which the plasma iron level is high, the morning mean plasma level was  $218 \pm 13 \mu\text{g}\%$ . This fell to a level of  $135 \pm 13 \mu\text{g}\%$  in the evening, a difference of  $83 \pm 10 \mu\text{g}\%$ . Similarly in 20 cases of anemia of

infection(11) in which the plasma iron level is usually low, the mean morning plasma iron level was  $56 \pm 6 \mu\text{g}\%$  and the evening value  $39 \pm 4 \mu\text{g}\%$ , representing a fall of  $17 \pm 4 \mu\text{g}\%$ . The diurnal rhythm appears to be related to activity and sleep since it has been shown by both Höyer(4) and Waldenström(6) that the diurnal variation is shifted in night workers, so that the level of plasma iron in these subjects is highest in the afternoon or evening after waking, and falls during the night, to rise again during the day when they sleep. Likewise, we have found that there was no definite diurnal cycle in a group of 5 normal individuals who were leading irregular hours of activity and sleep.

There is little evidence that muscular effort is a factor influencing the decrease in plasma iron during the day. Vannotti and Delachaux(12) stated that the plasma iron content decreases after heavy muscular effort but this conclusion is not apparent from the data they presented. Furthermore, Biörck (13) has been unable to confirm these findings.

Recent studies from this laboratory have demonstrated that there is a relationship between adrenocortical function and the level of plasma iron in dogs(14) and rats(15). It has been shown that the decrease in the plasma iron following acute stress is associated with

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increased adrenocortical secretion. Since Romanoff *et al.*(16) have observed that the excretion of adrenocortical steroids in man is greater during the morning and the rest of the day than during sleep, and that this excretion of steroids undergoes a constant diurnal variation, it might be postulated that the decrease in the level of plasma iron during the day may be associated with the increase in adrenocortical activity and similarly that the increase in the plasma iron during sleep may be associated with the decrease in the secretion of cortical hormone. However, it has been observed that the levels of eosinophils and lymphocytes in the peripheral blood increase rather than decrease as the day pro-

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gresses(17,18). Furthermore, two patients with Addison's disease whom we have studied showed a normal diurnal variation in the level of plasma iron. There is even less evidence to suggest that the decrease in plasma iron during the day is due to sympathetic tone and that its rise during the night is due to an increase in parasympathetic tone(8). Thus, the physiological mechanisms responsible for the diurnal variation in plasma iron of man remain obscure.

**Summary.** It has been shown that the plasma iron of man undergoes a regular diurnal variation. These results have been compared with those of other investigators.

We are indebted to Miss Helen Ashenbrucker for technical assistance as well as to the subjects who took part in this investigation.

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### Conversion of 3-Phosphoglycerate to Phosphoenolpyruvate by Tissue Homogenates.\* (18103)

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A series of recent observations(1-3) made it interesting to pursue the problem of regulatory mechanisms of enzyme reactions participating in the glycolysis of Harden-Young ester. The present paper is a part of this study and describes a method for the determination of phosphopyruvate formation from 3-phosphoglycerate, catalyzed by dilute tis-

sue homogenates. Evidence is also presented suggesting that this enzyme reaction might be under endocrine influence.

**Experimental.** Two enzymes, phosphoglyceromutase and enolase(4-6), are necessary to bring about the formation of phosphoenolpyruvate from 3-phosphoglycerate. Both enzymes are present in tissue homogenates. The principle of the assay of these two consecutive enzyme reactions is the determination of

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the formation of iodine labile phosphorus, which according to Lohmann and Meyerhof (4) and Meyerhof and Oesper(7) is a reliable measure of phosphoenolpyruvate. As pointed out by Lardy and Ziegler(8), alkali labile phosphates (triose phosphates) interfere with phosphopyruvate analysis. Since the possibility of triose phosphate formation from 3-phosphoglycerate in fresh tissue homogenates cannot be excluded, phosphopyruvate phosphorus was determined as the increment in inorganic phosphate, due to iodine treatment, above the inorganic phosphate present in the alkalinized samples. This procedure also includes the correction for inorganic phosphate, formed during the incubation period. The pH optimum for phosphopyruvate formation was found to be pH 6.8, which value agreed well with that reported by Warburg and Christian(6) for crystalline enolase in the presence of inorganic phosphate and  $Mg^{++}$  ion. The test was carried out in centrifuge tubes of 15 ml volume. The tubes containing the reaction mixture were incubated in a shaking device at 37°C for 10 minutes prior to the addition of the enzyme (0.1 ml tissue homogenate, equivalent to 1-5 mg tissue). Each centrifuge tube contained 0.5 ml 0.05 M potassium 3-phosphoglycerate† (brought to pH 6.8) + 0.5 ml trishydroxymethyl-aminomethane buffer (pH 6.8)(9) + 0.2 ml 0.05 M  $MgSO_4$  + 0.1-0.2 ml tissue homogenate in 0.9% KCl + 0.9% KCl to make a final volume of 1.5 ml. At the end of the incubation period the reaction was stopped by the addition of 1 ml of 1 N trichloracetic acid, the precipitated proteins centrifuged down at 2,000 r.p.m. in an International Centrifuge for 15 minutes. For the determination of phosphopyruvate phosphorus a 0.2 ml sample of the trichloracetic acid supernate was pipetted into each of 2 colorimeter cuvettes (Coleman spectrophotometer

Model 6-A). Thereafter 1 ml N KOH was added to each cuvette and 0.2 ml 0.1 N iodine solution ( $KI + I_2$ ) pipetted to one of the 2 tubes. The tubes were shaken and allowed to stand at room temperature for 15 minutes. To each cuvette 0.5 ml 5.0 N  $H_2SO_4$  was then added and in the iodine treated tube the discharged iodine decolorized by 0.2 ml 0.1 N Na-thiosulphate which was added, followed by vigorous shaking of the tube. This precaution was necessary to avoid occasional turbidity probably due to the precipitation of colloidal sulphur. In the tube which contained no iodine, equal volumes of distilled water were substituted for the iodine and thiosulphate. Finally the contents of each tube were made up to 3 ml by the addition of 0.9 ml distilled water. A reagent blank was always made up simultaneously, in which the 0.2 ml trichloracetic acid supernate was replaced by 0.2 ml  $H_2O$ . The determination of inorganic phosphate was carried out by the method of Gomori(10) because, under the condition of this method, the molybdate color reaction was not altered either by the acidity or by the iodine-thiosulphate treatment, as determined by comparing phosphorus values of iodine treated and untreated samples of a standard phosphate solution. The colorimetric phosphate analysis was carried out as follows: 2.5 ml molybdate- $H_2SO_4$  reagent and 1 ml of Elon-bisulphite solution(10) were pipetted to the contents of each colorimeter cuvette. After shaking the optical density was read in 45 minutes at 660  $m\mu$  on the Coleman spectrophotometer. The phosphopyruvate phosphorus was calculated from the reading difference between the alkali and alkali + iodine treated cuvettes from a phosphorus calibration curve. The iodine labile phosphorus read in micrograms, multiplied by 12.5, gives the total amount of phosphopyruvate phosphorus present in the 2.5 ml trichloracetic acid treated incubation mixture. This value can be converted to phosphopyruvic acid by multiplication by 5.9.

*Results and discussion.* The rate of phosphopyruvate formation was followed for 60

7. Meyerhof, O., and Oesper, P., *J. Biol. Chem.*, 1949, v179, 1371.

8. Lardy, H. L., and Ziegler, J. A., *J. Biol. Chem.*, 1945, v159, 343.

† Obtained as Ba salt from Schwarz Laboratories, 202 East 44th St., New York.

9. Gomori, G., *PROC. SOC. EXP. BIOL. AND MED.*, 1946, v62, 33.

10. Gomori, G., *J. Lab. Clin. Med.*, 1942, v27, 955.

PHOSPHOPYRUVIC ACID  
FORMATION BY RAT  
BRAIN HOMOGENATE

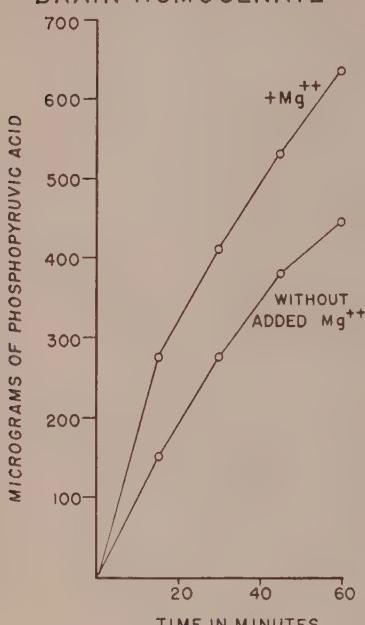


FIG. 1.

Enzyme = 5 mg male rat brain cortex homogenate.  $MgSO_4$  solution was replaced by 0.2 ml  $H_2O$ . The reaction was stopped at 15' intervals by the addition of trichloroacetic acid.

minutes using various tissue homogenates and extracts as the source of enzymes. A typical rate curve is presented in Fig. 1 where the activating effect of added  $Mg^{++}$  ions can also be observed. A linear relationship was found between the amount of enzyme (expressed in protein content of the tissue extract) and the amount of phosphopyruvic acid formed during a 15 minute incubation period (Fig. 2). Complete enzyme inhibition occurred when  $NaF$  was present in 0.01 M final concentration. The relative catalytic activity of various tissues could be compared by determining the phosphopyruvic acid formed by 1 mg tissue homogenate during 15 minutes incubation. Organs of 5 male and 5 female white rats, weighing between 250-340 g, were assayed by the described method. Results are summarized in Table I, where the mean values of determinations carried out

on separate organs of 5-5 rats, the standard error (S.E.), and also the "t" values(11) existing between the means of comparable male and female organs are shown. It is apparent that from animal to animal, reproducible values can be obtained on the same organs. The phosphopyruvate formation by tissue homogenates of female organs was significantly lower in all tissues except the adrenal as compared to male animals. Interestingly, the activity of ovaries significantly exceeds that of the testis ( $+50\%$  "t" = 3.66).

It is well known that the formation of phosphopyruvate and the transphosphorylation to the adenylic system(12-14) is an important step of glycolysis since it is directly

CORRELATION BETWEEN  
THE AMOUNT OF ENZYME AND  
RATE OF PHOSPHOPYRUVIC  
ACID FORMATION

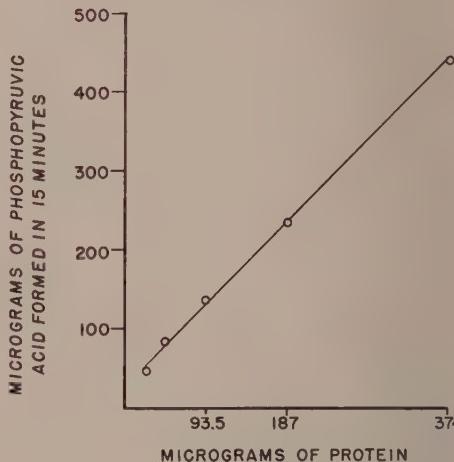


FIG. 2.

The enzyme was prepared by homogenizing 0.5 g rat brain cortex (male) in 10 ml 0.9% KCl and centrifuging at  $14,000 \times g$  for 20 min. at 0°C. Each test system (see text) contained 0.2 ml of the enzyme (supernate) which was serially diluted (1:1) with 0.9% KCl. The undiluted brain extract contained 374  $\mu$ g of protein per 0.2 ml.

11. Fisher, R., Statistical Methods for Research Workers, 1930, Oliver & Boyd, London.

12. Kubowitz, F., and Ott, P., *Biochem. Z.*, 1944, v317, 329.

TABLE I. Phosphopyruvic Acid Formation by Tissue Homogenates of Male and Female Rats.  
(Results expressed in terms of phosphopyruvic acid formed by 1 mg tissue during 15' incubation).

Organ	Phosphopyruvic acid				% difference	"t" values
	Male Mean	S.E.	Female Mean	S.E.		
Leg muscle	418.0 ± 19.80		258.8 ± 14.60		-38	6.5
Heart muscle	174.8 ± 24.70		100.1 ± 16.30		-43	8.55
Kidney	162.8 ± 17.6		72.7 ± 7.50		-55.5	4.72
Liver	58.1 ± 2.24		36.36 ± 3.38		-37.5	5.36
Brain	68.7 ± 5.55		44.17 ± 2.68		-35.7	3.96
Spleen	50.8 ± 7.00		31.72 ± 2.78		-37.6	6.35
Thyroid	72.4 ± 4.45		49.57 ± 2.00		-31.4	4.7
Adrenal	54.3 ± 9.53		38.92 ± 6.20		(-28.4)	1.35
Testis	45.3 ± 6.75					
Ovary	—		91.0 ± 10.5			
Uterus	—		78.0 ± 5.7			

S.E. = Standard error.

5% homogenates (0.1 ml) were used for enzyme assays of liver, brain, spleen, thyroid, adrenal, testis, and ovary; 1% homogenates (0.1 ml) for leg and heart muscle, kidney, and uterus.

concerned with storage of readily available energy in the form of energy-rich phosphate bonds. The marked differences found between male and female organs suggests that this energetically important phase of glycolysis might be regulated by hormones. Further data on the effect of endocrine organs on this enzyme reaction will be published in a subsequent paper.

**Summary.** It was shown that dilute homog-

enates of rat organs catalyze *in vitro* the conversion of 3-phosphoglycerate to phosphoenolpyruvate as measured by the formation of iodine labile phosphorus. A marked sex difference was observed between the rates of phosphopyruvate formation by male and female organs.

The valuable assistance of Mr. Donald R. McCurley, who carried out part of the enzyme assays, is gratefully appreciated.

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13. Bucher, T., *Biochem. et Biophysica Acta*, 1947, v1, 292.

14. Lipmann, F., *Adv. Enzymol.*, 1941, v1, 99.

## Properties of the Methanol Soluble Factor Required by the Mink.\* (18104)

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Previous work has shown that mink given a purified diet require a heat stable methanol

soluble liver factor, and preliminary results indicated that a methanol insoluble (residue) factor was also required(1). The methanol soluble factor was found in liver, whole milk and whey, but not in tomatoes, alfalfa or yeast. Similar results have been obtained with the fox(2). Further studies on these

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† Present address, Department of Animal Industry, North Carolina State College, Raleigh, N. C.

1. Schaefer, A. E., Tove, S. B., Whitehair, C. K., and Elvehjem, C. A., *J. Nutr.*, 1948, v35, 157.

TABLE I. Effect of Supplements to Basal Ration on Body Weight of Mink.

Mink No.	Ration	Before supplement		After supplement		Total wk on exp.	Body wt, g
		Total wk on exp.	Body wt, g	Supplement			
85	Basal	34	575	2% EtOH ext. of fish solubles		39	870
110	"	17	1055	4% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ppt of fish solubles		21	1260
111	"	23	1030	2% BuOH insoluble portion of fish solubles		26	1175
99	"	11	425	2% Acetone insoluble portion of MeOH ext.		19	710
142	" + 4% residue	5	595	4% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ppt of MeOH ext.		8	900
Avg for 117	" + 6% yeast + 8% residue	25	935	1% Phenol ext.		31	1230
140							
143							
Avg for 205	Basal	24	1060	10-20 µg vitamin B <sub>12</sub> /day		29	1290
235							
199							
208*							
Avg for 155	Basal + 1% MeOH ext.	36	905	4% Residue		50	1320
107							
178							
183							

\* High casein basal diet.

two factors are presented in this report.

*Experimental and results.* The mink were given the same basal ration used in the earlier work(3), with the exception that pteroylglutamic acid was included in the diet at a level of 100 µg per 100 g ration. The results obtained when various supplements were added to the basal ration are presented in Table I. It was found that the methanol extract factor was also present in fish solubles but not in 4% dried distillers solubles, dried whey or 2% desiccated hog intestinal mucosa. Fish solubles had been shown to contain a growth factor for chicks(4). Since the methanol extract factor is not present in dried distillers solubles it is unlikely that this factor is identical with vitamin B<sub>13</sub>(5).

2. Schaefer, A. E., Whitehair, C. K., and Elvehjem, C. A., *J. Nutr.*, 1948, v35, 147.

3. Tove, S. B., Schaefer, A. E., and Elvehjem, C. A., *J. Nutr.*, 1949, v38, 469.

4. Robblee, A. R., Nichol, C. A., Cravens, W. W., and Elvehjem, C. A., *Poultry Sci.*, 1947, v27, 442.

The chemical and physical properties of the methanol extract factor were studied further. An acetone precipitate of the methanol extract was found to be active. The methanol extract factor is insoluble in 3/4 saturated ammonium sulfate. Since an ammonium sulfate preparation was inactive at a 2% level, it would appear that at least 50% of the activity is lost by this procedure. A butanol soluble fraction obtained by continuous extraction of an 80% ethanol extract of fish solubles did not show activity. However, the butanol insoluble portion of a similar extract (extracted at pH 3 and 7) did produce a body weight response.

Phenol extraction of a methanol extract was accomplished in the following manner. Equal portions of the methanol extract and 90% phenol were shaken in a separatory funnel, the phenol layer removed, an equal volume of water added, and the phenol removed by

5. Novak, A. F., and Hague, S. M., *J. Biol. Chem.*, 1948, v174, 647.

ether extraction. This preparation proved as active as the methanol extract.

Previous work in this laboratory(3), has shown that the daily intramuscular injection of 1  $\mu\text{g}$  crystalline vitamin B<sub>12</sub> did not replace the methanol extract factor. Recently responses have been obtained by the daily injection of 10  $\mu\text{g}$  of crystalline vitamin B<sub>12</sub>, and by the oral administration of 20  $\mu\text{g}$  per day of this vitamin. In addition, a vitamin B<sub>12</sub> concentrate obtained from a streptomycin fermentation proved active when fed at a level equivalent to 10  $\mu\text{g}$  per day.

The results as shown in Table I confirm the earlier observation that a factor present in the methanol insoluble portion of liver (residue) is required by the mink. Poor fur development is one of the most prominent symptoms noted in animals which received only the basal ration. Animals which were given the residue fraction of liver as the only supplement were observed to have dense luxuriant underfur, equal to those receiving liver. Conversely the underfur of those mink which received only the methanol extract was extremely thin or nonexistent.

**Discussion.** It is apparent that striking similarities exist between the methanol extract factor required by mink and vitamin B<sub>12</sub>. It will be noted that there is a good correlation between the products containing methanol extract factor activity and their vitamin B<sub>12</sub> content (Table II). Furthermore the properties of the methanol extract factor and vitamin B<sub>12</sub> are very similar. The methanol extract factor is soluble in water, phenol and 60% methanol, but insoluble in acetone, n-butanol and 3/4 saturated ammonium sulfate. Vitamin B<sub>12</sub> has been shown to have similar properties(6). The classical symptoms of pernicious anemia, namely, anemia and neurological disorders, are seen in mink deficient in the methanol extract factor. Vitamin B<sub>12</sub> has been shown to be effective in preventing fatty degeneration of the liver and kidneys in the chick(7), and rat(8). The methanol

6. Smith, E. L., *British Med. J.*, 1949, v2, 1367.

7. Schaefer, A. E., Salmon, W. D., and Strength, D. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1949, v71, 202.

TABLE II. Comparison of Products Tested for Methanol Extract Factor Activity and Their Content of Vitamin B<sub>12</sub>.

Product	Methanol ext. factor activity	Min. vit. B <sub>12</sub> content (9), $\mu\text{g}/100 \text{ g}$
Liver	++	15
Fish solubles	++	20
Milk	++	2.5*
Hog intestinal mucosa	++	Trace
Pork spleen	+	†
Yeast	++	†
Distillers solubles	++	†
Alfalfa	++	†
Tomatoes	++	†

\* Whole milk powder.

† No measurable quantity.

extract cures a similar condition in the mink.

In spite of these similarities the methanol extract fraction must have an effect in addition to the actual amount of vitamin B<sub>12</sub> present as measured by rat assay(9). The methanol extract (which is active at an intake of less than 1 ml per day) contains only 2  $\mu\text{g}$  vitamin B<sub>12</sub> per ml while 10 to 20  $\mu\text{g}$  crystalline vitamin B<sub>12</sub> are needed to give the same activity. From the foregoing considerations, it would seem that the methanol extract factor may be another form of vitamin B<sub>12</sub>.

**Summary.** The methanol extract factor required by the mink has been found to be soluble in 90% phenol and insoluble in acetone, n-butanol and 3/4 saturated ammonium sulfate.

Fish solubles contain this factor, but dried distillers solubles, dried whey, pork spleen, and hog intestinal mucosa were found to be inactive.

The relationship between the methanol extract factor and vitamin B<sub>12</sub> is discussed.

The original observation that the mink requires a methanol insoluble factor as well as

8. Schaefer, A. E., Salmon, W. D., and Strength, D. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1949, v71, 193.

9. Lewis, U. J., Register, U. D., Tappan, D. V., and Elvehjem, C. A., unpublished data.

a soluble factor has been confirmed by the data presented.

We wish to acknowledge our indebtedness to Merck and Co., Rahway, N. J., for the crystalline

vitamins; to Lederle Laboratories, Pearl River, N. Y., for the pteroylglutamic acid.

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## Utilization of L-, D- and DL-Forms of Asparagine and Aspartic Acid by *Leuconostoc mesenteroides* P-60.\* (18105)

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A microbiological method for the determination of L-aspartic acid with *Leuconostoc mesenteroides* P-60 was reported by Hac and Snell(1). These authors showed that L-asparagine had only a fraction of the activity of L-aspartic acid for this organism, but although their method has been widely used it does not appear that the activities of D-aspartic acid and D-asparagine for *Leuconostoc mesenteroides* have been reported previously.‡ It seemed desirable, therefore, to determine the response of this organism to the optically active and racemic forms of aspartic acid and asparagine.

*Experimental.* The culture of *Leuconostoc mesenteroides* P-60 (American Type Culture Collection catalogue No. 8042) was the same as that described previously(2). The inoculum was prepared as described in Paper 47(3). The experimental medium and assay techniques were the same as those described previously for experiments with phenylalanine(4) except that 1 g of DL-phenylalanine was in-

cluded in the amino acid mixture of the basal medium and the L-asparagine was omitted. In some of the experiments the medium was modified by additions of sub-optimal amounts of L-asparagine or D-aspartic acid. Response to L-, DL-, and D-asparagines and aspartic acids§ was determined in the various modifications of the basal medium with incubation periods from 24 to 72 hours.

*Discussion.* Experiments with L-asparagine gave results which closely resembled those obtained previously by Hac and Snell(1). D-asparagine was entirely inactive in the present experiments (tested at final concentrations as high as 20 mg %), and DL-asparagine had 50% of the activity of L-asparagine.

Experiments with DL-aspartic acid gave essentially the same results (Fig. 1) as those obtained previously by Steele, *et al.*(5), however D-aspartic acid (Fig. 1) elicited a unique response. At concentrations up to about

\* Paper 74. For Paper 73, see Eiduson, *et al.*(4). This work was aided by grants from the American Cancer Society through the Committee on Growth of the National Research Council. The authors are indebted to Samuel Eiduson, Gene Molene, and Ruth B. Malin for technical assistance.

† Present address: Université de Liège, Laboratoire de Chimie Physiologique, Institut Léon Fredéricq, 17 Place Delcour, Liège, Belgium.

1. Hac, L. R., and Snell, E. E., *J. Biol. Chem.*, 1945, v159, 291.

‡ Steele, *et al.*(5) found that the activity of DL-aspartic acid was essentially the same as that of L-aspartic acid for *Leuconostoc mesenteroides*, but apparently these authors did not test D-aspartic acid.

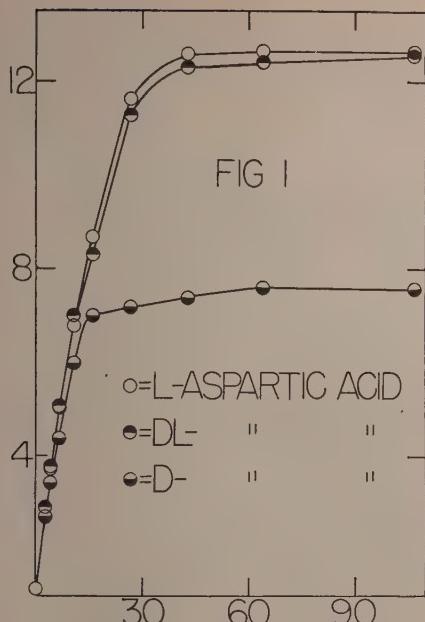
2. Dunn, M. S., Shankman, S., Camien, M. N., and Block, H., *J. Biol. Chem.*, 1947, v168, 1.

3. Dunn, M. S., Camien, M. N., Malin, R. B., Murphy, E. A., and Reiner, P. J., *Univ. Calif. Publ. Physiol.*, 1949, v8, 293.

4. Eiduson, S., Camien, M. N., and Dunn, M. S., *Paper 73, Archiv. Biochem.*, in press.

§ The L- and DL-asparagines and aspartic acids were A.P. and C.P. products of Amino Acid Manufacturers. The D-asparagine was a gift from M. Palmer Stoddard, Gerber, Calif. and D-aspartic acid ( $[\alpha]^{25.0} = -25.45^\circ$  in 1.008 N HCl) was obtained by acid hydrolysis of this product.

5. Steele, B. F., Sauberlich, H. E., Reynolds, M. S., and Baumann, C. A., *J. Biol. Chem.*, 1949, v177, 533.



Response of *Leuconostoc mesenteroides* to L-, DL-, and D-aspartic acids in the unaltered basal medium with 65 hours incubation. The values on the horizontal scale represent the concentration of aspartic acid in  $\gamma$  per ml. The values on the vertical scale are calculated as ml of 0.01 N NaOH required to titrate 1 ml of culture.

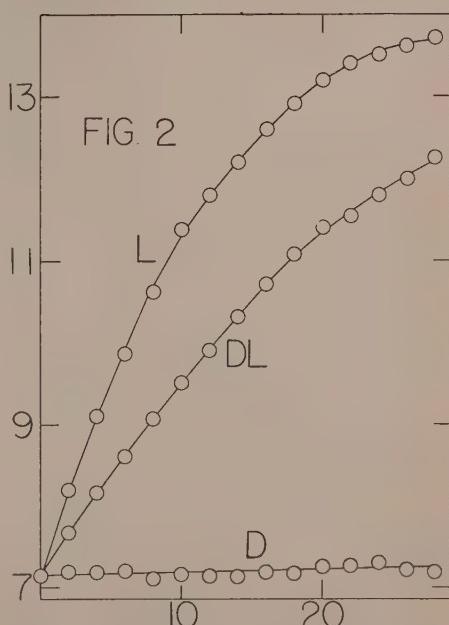
10  $\gamma$  per ml (Fig. 1) the activity of D-aspartic acid appeared to be nearly equal to that of L- or DL-aspartic acid. At higher concentrations of D-aspartic acid only a slightly increased response could be obtained. The response was not significantly greater than that shown in Fig. 1 at final concentrations of D-aspartic acid up to 20 mg %. It appears that a similar type of response to a D-amino acid by a lactic acid bacterium has not been reported previously.

It was found in further experiments that the relative activity of D-aspartic acid became progressively less with shorter incubation, and that with incubation periods as short as 24 hours D-aspartic acid activity was nearly negligible. DL-aspartic acid was only slightly less active than L-aspartic acid, even at short incubation periods.

When D-aspartic acid at a final concentration of 20 mg % was included in the basal medium (Fig. 2) the response to DL-aspartic

acid was approximately 50% of that to L-aspartic acid, and no significant response was obtained to further additions of D-aspartic acid. An incubation period of 48 hours was found to be nearly optimum for the demonstration of this effect. At longer incubation periods the blank titrations were excessively high, and at shorter incubation times the overall acid production was undesirably low. It seems probable that L-aspartic acid could be determined in samples containing relatively large amounts of D-aspartic acid with *Leuconostoc mesenteroides* under the conditions stipulated for Fig. 2.

Additions of L-asparagine to the basal medium markedly enhanced the response to D-aspartic acid (Fig. 3 and 4). D-asparagine appeared to be entirely inactive in producing this effect. A final L-asparagine concentration of 2.5 mg % appeared to be nearly optimal in producing approximately equal response to L-, DL-, and D-aspartic acids (Fig. 3). At lower L-asparagine concentrations the re-



Same as Fig. 1 except the basal medium was supplemented with D-aspartic acid (final concentration, 20 mg %), and the incubation period was 48 hours.

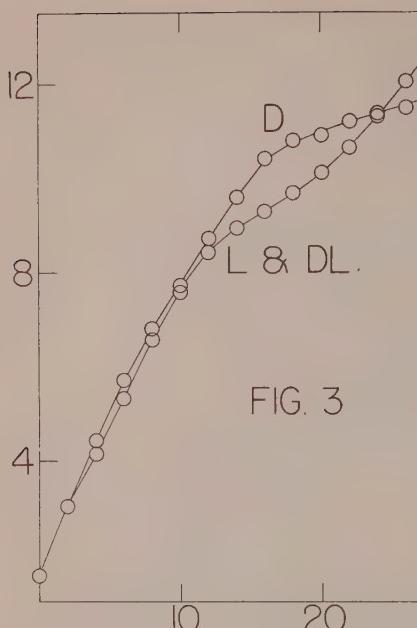


FIG. 3

Same as Fig. 1 except the basal medium was supplemented with L-asparagine monohydrate (final concentration, 2.5 mg %), and the incubation period was 72 hours. The L- and DL-aspartic acid curves are superimposed. The deviations did not exceed the radii of the plotted circles.

sponse to D-aspartic acid tended to level off before the maximum acid production was obtained, and at higher concentrations high blank titrations and relatively high response to D-aspartic acid resulted (Fig. 4). It seems likely that total (L- and D-) aspartic acid could be satisfactorily determined with *Leuconostoc mesenteroides* under the conditions stipulated for Fig. 3, if the test range were limited to aspartic acid concentrations below 12 γ per ml. (The activity of D-aspartic acid appears to be significantly greater than that of L-aspartic acid at concentrations between 12 and 24 γ per ml.)

The facts that D-aspartic acid under certain conditions (Fig. 4) is more active than L- and DL-aspartic acids and that L-asparagine seems to have some function, particularly in the utilization of D-aspartic acid, indicate that D-aspartic acid and L-asparagine may be essential metabolites for *Leuconostoc mesenteroides* even though these compounds are

less active than L-aspartic acid under the test conditions which are usually employed. The role of D-alanine(6-8) and D-glutamic acid(9) as essential metabolites for lactic acid bacteria has been suggested previously.

**Summary.** The utilization of the optically active and racemic forms of asparagine and aspartic acid by *Leuconostoc mesenteroides* has been studied under a variety of test conditions. D-asparagine was found to be entirely inactive, and DL-asparagine was found to have 50% the activity of L-asparagine. Conditions were described under which D-aspartic acid was essentially inactive and under which DL-aspartic acid had 50% the activity of L-aspartic acid. Under other con-

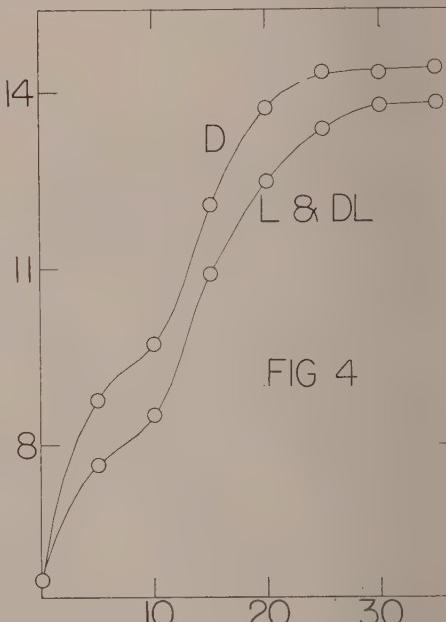


FIG. 4

Same as Fig. 3 except the final concentration of L-asparagine hydrate in the basal medium was 5.0 mg %.

6. Holden, J. T., Furman, C., and Snell, E. E., *J. Biol. Chem.*, 1949, v178, 789.
7. Holden, J. T., and Snell, E. E., *J. Biol. Chem.*, 1949, v178, 799.
8. Camien, M. N., and Dunn, M. S., *J. Biol. Chem.*, 1950, v185, 553.
9. Dunn, M. S., Camien, M. N., Rockland, L. B., Shankman, S., and Goldberg, S. C., *J. Biol. Chem.*, 1944, v155, 591.

ditions L-, DL-, and D-aspartic acids had nearly equal activities. Methods for the separate microbiological determination of L-aspartic acid and total (L- and D-) aspartic

acid with *Leuconostoc mesenteroides* were suggested.

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### Effect of Phlorhizin on Intestinal Absorption of Glucose, Galactose, Fructose, Mannose, and Sorbose. (18106)

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Although phlorhizin has repeatedly been shown to inhibit the intestinal absorption of glucose(1-8), the extent of inhibition reported has varied from 30%(7) to 90%(1). The effect of this glucoside on the absorption of hexoses other than glucose has not been investigated as thoroughly. Wertheimer(6), using the Cori technic, measured the absorption of glucose, galactose, fructose, and mannose by pairs of unanesthetized rats. One rat of each pair received an alkaline solution of 100 mg of phlorhizin in sugar solution by gastric tube; the control received only the sugar solution. Although he used only 2 pairs of rats each to examine fructose and galactose, and only one pair for mannose, he concluded that the inhibition exerted by phlorhizin upon glucose absorption applied also to the absorption of galactose, fructose, and mannose. Yoshikawa(8), using 2 consecutive 30 cm seg-

ments of rabbit jejunum, introduced 5.4% solutions of various sugars into these loops and removed samples for analysis at regular intervals. By putting into one of the loops a solution of phlorhizin, sugar, and sodium bicarbonate, and into the other loop only the sugar and bicarbonate, he was able to use the same intestine both for control and experiment. He found that the order of absorption for the sugars tested was: galactose, glucose, mannose, arabinose, xylose, and fructose. The greatest phlorhizin inhibition appeared to be upon galactose absorption, the least upon fructose.

Cori(9) and Wilbrandt and Laszt(10) have found glucose and galactose to be rapidly absorbed, mannose and the pentoses to be absorbed quite slowly, and the absorption rate of fructose to be intermediate between that for glucose and that for mannose. Wertheimer's(6) data do not include the various rates of absorption, since he measured the percentage of sugar absorbed. Yoshikawa's (8) finding that fructose was absorbed more slowly than mannose, or even xylose, suggested the need for further experiments along this line.

**Experimental.** The experiments were performed on albino and hooded rats of both sexes selected from stock raised in this laboratory. Absorption was studied by means of a technic employing the entire small intestine. Prior to the experiment the rats received 6 subcutaneous injections of a 10% suspension

\* This investigation was performed in partial fulfillment of the requirements for the degree of Master of Science in the Department of Physiology, State University of Iowa.

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of phlorhizin in sesame oil (each dose being 0.25 cc/100 g) at intervals of 12 hours. The control animals, which were paired with the experimentals for sex and strain, received similar doses of the oil alone. A few rats were not injected with oil; since this group did not differ from the oil-injected controls, the data on both groups were pooled as controls. For 2 days before the experiment the rats received no solid food in order to clear the intestinal tract of reducing material, but were given a low residue meal of 3.3 cc of evaporated milk/100 g body weight, by gastric tube, one day preceding the experiment. The rats also received water by stomach tube at 12 hour intervals during the fast in order to further promote removal of food and debris from the intestine. At the end of this time a sample of urine was tested with Benedict's qualitative reagent(11) and showed in every phlorhizinated animal a copious glycosuria, while the control urines were always negative for sugar.

Each rat was then anesthetized with an intraperitoneal injection of Nembutal (45 mg/kg), the abdomen opened, and ligatures placed at the pyloric and caecal ends of the small intestine. The rats were kept warm (rectal temperature about 38°C) during the procedure. A measured volume (according to the animal's weight) of a 5.5% solution of the hexose being studied was injected through the wall of the ligated gut at several sites, starting at a fixed time after the Nembutal injection, so that all of the animals were at the same stage of anesthesia during the absorption period. The abdomen was then closed until 25 minutes later when the intestine was excised, sliced open, and washed with its contents into a 500 ml volumetric flask where the contents were precipitated using the zinc hydroxide procedure of Somogyi(12). An aliquot of the protein-free filtrate was analyzed according to the Nelson (13) colorimetric modification of Somogyi's (14) method for slowly reacting sugars. Ab-

sorption was determined as the difference between the amount of sugar administered and the amount recovered. The rate of absorption was calculated [after Cori(9)] as the coefficient of absorption: mg absorbed/100 g body weight/hour.

The data were analyzed statistically by the use of Student's *t*, a method which has been recommended for small samples. The standard deviation was calculated as:

$$\sigma_x = \sqrt{\frac{\sum x^2}{N} - \left(\frac{\sum x}{N}\right)^2}$$

The *t* was found from the formula

$$t = \frac{M_x - M_y}{\sqrt{\left(\frac{\sigma_x^2 N_x + \sigma_y^2 N_y}{N_x + N_y - 2}\right) \left(\frac{N_x + N_y}{N_x N_y}\right)}}$$

*M* representing the mean, and *N* the number of units in the samples. The value of *t* gives the level of confidence for the difference *M<sub>x</sub>*—*M<sub>y</sub>*, taking into account the size of the samples(15).

*Results.* The data are presented in Table I. The mean coefficients of glucose absorption were 116.6 for the control rats and 72.0 for the phlorhizinated animals, a decrease of 38.2%. The level of confidence of the experimental difference, determined by the use of Student's *t*, is over 99.9%, indicating that there was a very significant inhibition of glucose absorption by phlorhizin.

Galactose absorption was reduced from a mean coefficient of 101.2 to 55.7 by phlorhizination. This inhibition was as significant as that of glucose. The degree of phlorhizin inhibition of galactose absorption (44.0%) was roughly the same as that of glucose.

Mannose absorption was reduced from a coefficient of 19.9 to 14.6 by phlorhizin, an inhibition of 26.6%. The data indicate some phlorhizin inhibition although the statistical reliability of this difference is poor (level of confidence only 33.4%). Sorbose was normally absorbed at such a slow rate, 8.3 mg/100 g/hour, that the differences in reducing

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TABLE I. Coefficients of Absorption of 5 Hexoses for Phlorhizinized and Control Rats.

Sugar	Control rats				Phlorhizinized rats				Effect of phlorhizin	
	No. rats	Coeff. of absorp. $\sigma$	% of glucose	No. rats	Coeff. of absorp. $\sigma$	% of glucose	% of inhibition	Level of confidence of diff., %		
Glucose	18	116.6 (77-171)*	28.5	100.0	15	72.0 (54.5-100)	19.1	61.8	38.2	99.9
Galactose	23	101.2 (35-170)	28.6	86.9	25	55.7 (2-126)	31.8	47.8	44.0	99.9
Fructose	13	49.3 (18-77)	20.0	42.4	14	63.4 (25-88)	17.5	54.3	-28.6	92.8
Mannose	6	19.9 (6-37)	10.9	17.1	5	14.6 (1.5-27.5)	9.1	12.5	26.6	†
Sorbose	6	8.3 (1-20)	7.5	7.1	5	0.9 (0-2.5)	0.9	0.8	90.0	†

\* The figures in parentheses indicate the range of coefficients found.

† Statistical analysis of samples of this size would be unreliable.

substances were within experimental error range. The effect of the phlorhizin was exaggerated with the small number of animals used and statistical evaluation would be inadequate.

The results for fructose absorption were in striking contrast to those for the other 4 hexoses. The phlorhizinized rats absorbed fructose at a rate 28.6% greater than the controls, the drug appearing to have increased rather than inhibited fructose absorption. The statistical significance of this increase is not high (level of confidence 92.8%), but at least there is no doubt that the drug failed to inhibit fructose absorption.

**Discussion.** The relative order of rates of absorption in the control rats compares favorably to that reported by Cori(9) and by Verzár(16), as shown in Table II. The absorption of galactose averaged 13% less than that of glucose in these experiments, but statistical evaluation assigns a low level of confidence to this (91%). The absorption rates, while in nearly the same relative order as those reported by Cori for unanesthetized rats, were actually 30-40% lower. This, together with

some preliminary observations not reported here, has led us to believe that Nembutal anesthesia exerts a general depression on hexose absorption without altering the relative rates of absorption of the sugars to each other.

Gammeltoft and Kjerulf-Jensen(17) have suggested that phlorhizin inhibits hexose absorption by blocking some enzyme necessary for the phosphorylation, and hence absorption, of glucose, galactose, and fructose. They have further postulated that the enzyme inhibited is the phosphate donor, adenosinetriphosphatase, rather than the hexose phosphorylase. The failure of phlorhizin to inhibit fructose absorption in these experiments makes it seem much more probable that it is the phosphorylase that is inhibited by phlorhizin, and suggests that separate enzymes may exist in the mucosa for the phosphorylation of each of the hexoses. The fructose phosphorylase would appear not sensitive to the inhibitory action of phlorhizin. The existence of specific phosphorylating enzymes is further supported by the recent finding of such specific enzymes as fructokinase in muscle(18) and galactokinase in liver and yeast(19).

**Summary.** Phlorhizin has been found to

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TABLE II. Intestinal Absorption of Hexoses Referred to Glucose as 100%.

	These data	Cori(9)	Verzár(16)
Glucose	100	100	100
Galactose	87	110	115
Fructose	42	43	44
Mannose	17	19	33
Sorbose	7	—	30

inhibit the intestinal absorption by rats of glucose, galactose, and possibly of mannose and sorbose, but not of fructose. This may indicate the presence in the intestinal mucosa

of a specific enzyme for fructose phosphorylation which is not inhibited by phlorhizin.

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### Observations on the Toxin of Influenza Virus.\* (18107)

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Henle and Henle(1) reported the demonstration of a toxin associated with influenza virus and later studied this factor more extensively(2-4). During an investigation in our laboratory of further toxic manifestations of the virus on the circulatory system of the host(5,6) difficulties in maintaining the toxin-producing properties of the virus were encountered; therefore, it was felt desirable to repeat and extend some of the experiments of Henle and Henle. Only the PR8 strain<sup>†</sup> of influenza A virus was used. With careful attention to conditions of storage of the virus, consistent production of toxin was attained. In general, our observations of the lesions produced by the toxin, the relation of toxin to other established virus properties, its production and stability and host factors affecting toxicity have confirmed and amplified the findings of Henle and Henle, with a few differences. In addition, the susceptibility of the

ferret to influenza virus toxin was demonstrated.

*Methods.* Toxin of the influenza virus was produced by inoculating the allantoic sac of 10 to 11 day chick embryos with 0.15 ml of a  $10^{-6}$  dilution of infected allantoic fluid in brain-heart infusion broth. The eggs were then incubated at 37°C for 48 hours, after which they were chilled before harvesting the allantoic fluids. The fluids were tested for bacterial contamination before pooling. The allantoic fluid was tested for toxicity by injecting 1.0 ml amounts intravenously into the tail vein of 12 to 15 g CFW mice. Dilutions for intravenous injection were made in normal allantoic fluid. When virus was purified and concentrated it was done by adsorption and elution from red blood cells followed by high speed centrifugation(5). Virus was tested for infectivity by injecting 0.15 ml of 10-fold dilutions of viral suspensions into 10 to 11 day embryonated eggs. The presence of virus in fluids harvested from these eggs was determined by the hemagglutination technic using the method of Salk(7) with the exception that the concentration of the chick red cell suspension used was 0.5 instead of 0.25%.

*Experimental.* As the Henles have shown, the intravenous injection of allantoic fluid containing PR8 virus produced toxic manifestations consisting of gross lesions of the liver, spleen, frequently the intestines, the kidney, and engorgement of pulmonary vessels without parenchymal consolidation. In our experiments, alterations in some or all of

\* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, United States Public Health Service.

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† This toxin-producing strain was kindly sent to us by Dr. Werner Henle.

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these organs were observed in 92% of 352 mice. Microscopically, representative sections of these lesions showed hemorrhagic necrosis.<sup>†</sup> The relationship between the amount of virus injected and the time of death after injection indicated that our toxin was less potent than that prepared by Henle and Henle(3), since the maximum number of deaths did not occur until 48 hours after injection of the toxin, rather than within 24 hours. Further, their toxin in a dilution of 1:4 regularly killed mice on intravenous injection while less than 5% of 251 mice died following the injection of a 1:4 dilution of various preparations of our toxin. The toxic activity was neutralized by specific anti-serum. The Henles noted in a few instances that moribund mice suspended by their tails reacted with convulsions, and a few animals were observed in spontaneous convulsions; these findings were also observed in our mice.

In their observations on the lungs Henle and Henle reported only engorgement of the pulmonary vessels; the concentration of virus in the lung tended to remain constant in contrast to a marked fall in virus concentration in the liver and peritoneal fluid after intra-abdominal injection. In our experiments pulmonary consolidation occurred even in mice which died within a period in which the deaths might have been due, at least in part, to the action of the toxin. It seems unlikely that virus could have invaded the lung and multiplied so rapidly that the early consolidation was due to infectivity alone. Of 75 mice which died within 24 hours, 8% had at least 50% pulmonary consolidation; of 134 mice which died between 24 and 48 hours, 20% had consolidation. Combining these data, 15% of the mice which died within 48 hours after intravenous injection had pulmonary consolidation. Of 33 mice which died between 5 and 10 days after injection, when infection by the virus had taken place, 79% had pulmonary consolidation. On investigating the relation of other properties of the virus to the toxic agent, Henle and Henle ob-

TABLE I. Relationship of Virus Inoculum to Infectivity and Mouse Toxicity of Harvested Virus

Dil. of inoculum	Titration of infectivity in embryonated eggs				Intrav. toxicity for mice	
	10-7	10-8	10-9	10-10	Undil.	1:4
10-1	3/5*	0/5	1/5	0/2	0.8	0/3
10-3	5/5	5/5	2/5	0/2	7/8	0/6
10-4	5/5	3/5	1/5	0/2	8/8	0/7
10-5	6/6	6/6	3/6	1/3	5/8	0/7
10-6	6/6	4/6	1/6	0/3	8/8	3/7
10-7	6/6	6/6	2/6	0/3	8/8	5/7
10-8	6/6	5/6	0/6	0/3	1/8	0/3
10-9	0/3	0/3	0/3	0/3	1/4	—

\* Numerator = No. of mice dead.

Denominator = No. of mice inoculated.

served that, as in the production of infective virus and the hemagglutination factor, a dilute inoculum produced preparations of greater toxicity than more concentrated seed. They found that there was a correlation between infectivity titer and toxicity. This correlation was not absolute, however, as differences in time of appearance and duration of maximum titers indicated.

In Table I are recorded the results of an experiment in which groups of 6 to 8 eggs were inoculated with dilutions of virus ranging from 10<sup>-1</sup> to 10<sup>-9</sup>. After 48 hours, the allantoic fluids of each group were pooled and tested for chick embryo infectivity and for toxicity. The harvest from the initial inoculum of 10<sup>-1</sup> presumably demonstrated the interference phenomenon both for infectivity and toxicity. When the initial inoculum ranged from 10<sup>-3</sup> to 10<sup>-8</sup>, the infectivity titer of the fluids, all harvested at 48 hours, was 10<sup>-8</sup> or slightly higher in each case; the toxicity titer, however, was greatest when the initial inoculum was 10<sup>-6</sup> or 10<sup>-7</sup>. Injection of toxin harvested from eggs which received an initial inoculum of 10<sup>-8</sup> produced only one death. The reason that optimum toxin production occurred with a highly dilute inoculum is unknown at present. Although attempts to separate the toxin from the infective portion of the virus molecule have been unsuccessful(3), the findings of Henle and Henle and those here reported indicate that there may be different fractions of the virus, or that qualitatively different virus may exist. An alternative explanation would depend on

† We are indebted to Dr. Cecil Krakower for making the microscopic examinations of the tissues studied.

TABLE II. Relation of Toxin to Hemagglutination Titer of Virus.

Hemagglutination titer of virus	No. of mice injected	% mortality
1:6400	4	100
1:3200	102	87
1:1600	122	80
1:800	53	72
1:400	8	13

the demonstration of an inhibitor of toxin.

In Table II it may be seen that there is a correlation between the hemagglutination titer of the virus and the toxic property. Virus with a titer of 1:400 was of low toxicity; as the hemagglutination titer increased the toxicity also increased, although not proportionately. The Henles' findings also suggested a rough correlation with hemagglutination titer.

Since there is some indication that the toxic fraction may be a separate entity of the virus molecule, a study was made to determine whether there was an optimal incubation temperature of the inoculated eggs which would result in maximal toxin production. Between 33 and 37°C, the toxin production and infectivity were relatively constant. Outside this range (31 to 39°C), the toxicity was greatly decreased but the hemagglutination titer was also very low so that there was no indication of the toxic factor's being produced under temperatures different from those which were optimum for the virus as a whole.

Genetic variation of the mouse host might also be expected to be of importance in the manifestation of toxicity. Testing 4 strains of mice, the Henles(4) found that the LD<sub>50</sub> varied with the strain, although the data indicate that the differences were small (LD<sub>50</sub> 1:3.7 and 1:5.7). In our experiments, 10 strains of mice were tested; all were males weighing from 10 to 15 g. These included CFW, Harlan, Rockland, DBA, C-57, A-K Leukemia, C<sub>3</sub>H, fostered C<sub>3</sub>H, CFL, and stock white mice. As seen in Table III no significant difference in susceptibility was noted among these strains in the dilutions tested.

The Henles concluded that younger mice were more susceptible to the toxic activity of the virus. They injected 1.0 ml amounts

of the undiluted or diluted toxin preparations. In our tests, the amount of inoculum was 1.0 ml per 15 g of mouse body weight. Under these conditions, the 3- to 4-week-old mice were somewhat more susceptible than 6- to 12-week-old mice. On testing the virus in the ferret (*Putorius putorius*) it appeared that the virus was also toxic to this species. In Table IV it will be observed that a virus preparation containing 154 hemagglutinating units per g of body weight was required to kill 6 of 8 animals within 18 hours, while 46.5 units per g were on the borderline of toxicity. Material with 10.1 units per g of body weight produced no deaths and no gross or microscopic abnormalities with the exception of an enlarged liver and spleen in one ferret. Animals receiving the larger amounts of virus (Groups 2 and 3) showed enlargement of the spleen and occasionally of the liver if they survived for 24 hours; ferrets dying in less than 24 hours showed no gross changes, but microscopically congestion and infiltration with polymorpho-

TABLE III. Susceptibility of Various Strains of Mice to Toxin.

Strain	Intrav. inj. of virus undiluted 1:4	
CFW	10/10	2/10
Harlan	9/10	1/10
Rockland	8/10	0/10
DBA	8/10	0/10
C-57	10/10	0/10
A-K leukemia	9/11	0/11
C <sub>3</sub> H	11/11	0/11
C <sub>3</sub> H fostered	11/11	1/11
CFL	4/4	0/4
Stock white mice	6/11	0/11

TABLE IV. Relationship Between Hemagglutination Titer of Influenza Virus and Its Toxicity for Ferrets.

Group No.	Hemagglutination units per g of body wt	Mortality ratio
1	10.1	0/5*
2	46.5	1/4
3	154.0	6/8

\* Numerator = No. of ferrets which died.  
Denominator = No. of ferrets which were injected.

§ Hemagglutinating units per g =  
Hemagglutination titer x ml injected  
g of body wt

nuclear cells were observed in the liver, spleen and adrenal glands. These changes were not considered sufficient to cause death and it was believed that the toxin in high concentration was capable of acting on vital centers before significant demonstrable lesions had occurred. On comparing the toxicity of the virus for mice and ferrets, it may be observed that virus with a hemagglutination concentration of 154 units per g of body weight was required to kill 75% of the ferrets, whereas 53 units per g of body weight were required to kill approximately 75% of the mice. Thus it appeared that mice were more susceptible to the toxin than ferrets.

A knowledge of the stability of the virus is of obvious practical importance. The Henles found that toxicity was unaltered for 1 to 3 months at 4°C, decreasing slowly thereafter. In our experiments, the toxicity was very slight after periods of longer than one week at 4°C; this was true whether the virus was preserved unpurified and unconcentrated in allantoic fluid or in the purified

and concentrated state in 0.1 M phosphate buffer solution at pH 7.0. However, there was no decrease in toxicity in material stored in allantoic fluid for 3 months at -30°C or at -60°C. On two occasions our stock virus lost its capacity to stimulate production of the toxic factor on inoculation into eggs while it retained its ability to produce the hemagglutination and infectivity factors when it had been stored for periods of 6 months at -60°C. This loss of toxin production was also observed on one occasion on serial passage in the embryonated egg. These findings lend further evidence to the hypothesis that the toxic factor is different from the infective moiety.

*Summary.* The mouse toxic factor of influenza virus, PR<sub>8</sub> strain, originally reported by the Henles was reinvestigated, and similarities and differences between their observations and ours are reported and discussed. The toxin also was shown to affect ferrets.

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## Excretion of Phenylalanine and Derivatives in Phenylpyruvic Oligophrenia. (18108)

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It is established that patients affected with phenylpyruvic oligophrenia excrete in the urine phenylalanine(1,2), phenylpyruvic acid (2-8), and phenyllactic acid(1,2,6). Quantitative determinations of phenylpyruvic acid in a few patients have been published(2,4,5),

but only in one patient were data reported on the excretion of these 3 phenyl compounds (2). It is the purpose of this note to present quantitative data on the urinary excretion of phenylalanine, phenylpyruvic and phenyllactic acid in 20 patients, under varying dietary conditions.

*Materials and methods.* Twenty individuals affected with phenylpyruvic oligophrenia ranging in chronological age from 2 to 42 years and in the intelligence quotient from 5 to 47, were used as experimental subjects. For the determination of free phenylalanine

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10 ml of urine were diluted to 50 ml and extracted with chloroform for 24 hours to remove phenylpyruvic and phenyllactic acids. The chloroform was evaporated and phenylalanine was determined on 5, 4, 3, 2, and 1 ml of the urine diluted from 20 to 100 times following the microbiological assay with *Leuconostoc mesenteroides* P-60 described by Dunn *et al.*(9). Five ml of medium was used for each test and the determinations were performed titrimetrically with 0.04 N sodium hydroxide after 5 days incubation period. A standard curve was prepared, using DL-phenylalanine tested for purity.\* Calculations were made on the assumption that all urinary phenylalanine is of the L form, as maintained by Fölling *et al.*(1) and confirmed by Borek and Waelsch(10).

For the determination of phenylpyruvic acid, the "direct total hydrazone" method of Friedman and Haugen(11) was used on aliquots of the original urine diluted from 20 to 50 times. A standard curve was prepared using recrystallized phenylpyruvic acid prepared according to Hemmerlé(12). The colorimetric determinations were performed in a Klett photoelectric apparatus using Filter 54.

For the determination of phenyllactic acid, the nitration method of Kappeler-Adler(13), previously reported(14), suitable for this acid, was used in the following manner. To 2-5 ml of urine, 2 ml of a saturated solution of sodium bisulphite was added. Phenylpyruvic acid was thus bound into a chloroform insoluble compound. After 10 minutes the acidified solution was extracted 3 times with

4 ml of chloroform for 30 minutes. The combined chloroform extracts were added to 5 ml of N sulfuric acid and evaporated. Nitration and development of color were performed following the directions of Roche and Michel(15) and using an electric stove as recommended by Albanese(16). Readings were performed in a Klett photoelectric apparatus with Filter 54. A standard curve was prepared using twice recrystallized calcium salt of phenyllactic acid prepared according to the directions of Fischer(17). A blank was run with each determination using the same ingredients but ascorbic acid. Fairly satisfactory recoveries, within 10% error, were obtained upon applying this procedure to a mixture of phenyllactic, phenylpyruvic acid and phenylalanine.

Nitrogen was determined with the macro-Kjeldahl method using a digesting mixture containing selenium.

*Results and discussion.* Table I shows the results of determinations of phenylalanine, phenylpyruvic acid and phenyllactic acid in 20 patients on normal institutional diet. The values are expressed in mg per g of total urinary nitrogen. This manner of notation was necessarily adopted, since it was impossible in many patients of low mentality to collect complete 24 hour specimens of urine. The urinary output of phenylalanine was found to vary from 50 to 20 mg per g of total nitrogen, phenylpyruvic acid from 170 to 112 mg, and phenyllactic from 91 to 40 mg. No conspicuous differences in the output of phenyl compounds were observed from patient to patient, suggesting that in contrast with other errors of metabolism, only small differences in the degree of metabolic defect occur.

It is significant to note from Table I the lack of correlation between intelligence quotient and amount of urinary phenyl compounds. Were there a causal relationship of

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TABLE I. Urinary Output of Phenyl Compounds on Normal Diet.

Patient	Age	I.Q.	Phenylalanine, mg/g N	Phenylpyruvic a., mg/g N	Phenyllactic a., mg/g N	Total phenyl- compounds, mg/g N
A.	32	7	26	166	66	258
B.	42	46	22	120	68	210
C.W.	26	7	50	145	91	286
C.B.	27	40	25	159	71	255
D.	19	9	34	170	65	269
F.	28	5	35	149	65	289
G.	29	5	33	130	45	208
K.R.	5	24	20	116	52	188
K.A.	30	38	35	114	72	221
K.A.	22	6	20	144	69	233
K.P.	2	23	30	160	42	232
K.W.	34	26	25	150	72	247
M.G.	20	7	20	137	65	222
M.E.	15	35	28	112	48	188
M.R.	26	10	49	139	40	228
M.M.	41	11	35	140	90	265
M.S.	17	12	20	122	51	193
N.	23	7	33	113	61	207
R.	29	47	28	181	90	309
Z.	6	14	25	180	46	251

the metabolic abnormality to the mental defect, as suggested by Fölling *et al.*(18), one would expect that the lower the intelligence quotient the higher the amount of excreted phenyl compounds.

Table II shows the amount of phenyl compounds excreted in 24 hours by one patient on a high and on a low protein diet. It demonstrates the dependence of the daily output of phenyl compounds on the amount of protein ingested. However, that not all phenyl compounds derive from dietary protein was indicated by the observation that two patients still excreted phenylalanine and its keto and hydroxyl acids after 10 days of protein free diet.

The effect of various amino acids on the

TABLE II. Urinary Output of Phenyl Compounds on High and Low Protein Diet.

Date	N, mg/24 hr	Phenylalanine, mg/24 hr	Phenylpyruvic acid, mg/24 hr	Phenyllactic acid, mg/24 hr
3-29	22.000	440	2289	1080
4-6*	4.010	76	428	208

\* Very low protein diet started on 3-30.

18. Fölling, A., Mohr, O. L., and Ruud, L., Oligophrenia phenylpyruvica, Oso, 1945.

TABLE III. Urinary Output of Phenyl Compounds Following Ingestion of Phenylalanine and Phenylpyruvic Acid.

Date	N, mg/24 hr	Phenylalanine, mg/24 hr	Phenylpyruvic acid, mg/24 hr	Phenyllactic acid, mg/24 hr
4-26	15250	323	1640	1060
4-27*	16330	664	5840	1620
3-22	10.20	324	1380	680
3-23†	11.04	348	4200	2000

\* Phenylalanine g 10.

† Phenylpyruvic acid g 10.

excretion of phenyl compounds was tested by determining the daily output in patients kept on a nitrogen constant diet following ingestion of some pure amino acid. The following amino acids produced no change: glycine (25 g); glutamic acid (5 g); tyrosine (10 g); alanine (10 g); cystine (10 g). As seen in Table III, the ingestion of DL-phenylalanine (10 g) resulted in an increased excretion of all phenyl compounds. In this experiment, however, a certain amount of the keto acid probably originated from the D phenylalanine. The ingestion of 10 g of phenylpyruvic acid (Table III) resulted in a significant increase in the excretion of both the keto and the hydroxy-acid. In normal

TABLE IV. Excretion of Phenylalanine and Phenylpyruvic Acid in the Sweat (mg/100 cc).

Patient	N, mg/100 cc	Phenylalanine, mg/100 cc	Phenylpyruvic acid, mg/100 cc
A.	61	2.3	20
B.	100	3.4	15
C.	57	1.6	19
F.	44	1.6	6
K.P.	54	1.4	13
K.A.	56	2.4	56
M.M.	76	2.7	25
M.E.	140	4.4	33
N.	84	3.5	21
R.	152	9.6	47

individuals this amount of phenylpyruvic acid is usually completely metabolized.

From these feeding experiments it would appear that some quantitative changes in the excretion of phenyl compounds should be expected when foods with different phenylalanine content are ingested by the patients. However, since the ratio of phenylalanine to nitrogen in various proteins and foods does not show conspicuous variations(19), the amount of phenyl compounds excreted per g of nitrogen, as expressed in Table I, is probably little affected by the quality of ingested food.

In view of the findings of urinary excretion of phenyl compounds, it was considered of interest to investigate the excretion of

19. Horn, M. J., Jones, D. B., and Blum, A. E., *J. Biol. Chem.*, 1948, v176, 679.

these metabolites in sweat. Accordingly, sweat was collected as directed by Hier *et al.* (20) from 10 patients and determinations were performed on suitable dilutions following the procedure described for urine. The patient was bathed before the collection of sweat. Phenyllactic acid was not found in measurable amounts. The data concerning phenylalanine and phenylpyruvic acid are shown in Table IV. The values for the amino acid were somewhat above those reported by Hier *et al.*(20) in the normal individual. Phenylpyruvic acid is not found in the sweat of normal subjects. It would appear, therefore, that a small amount of phenylalanine and its derivative is eliminated by way of sweat.

*Summary.* Quantitative data are presented on the urinary excretion of phenylalanine, phenylpyruvic acid and phenyllactic acid in twenty patients affected with phenylpyruvic oligophrenia. The values, expressed in mg per g of total nitrogen, show inconspicuous differences from patient to patient kept on similar diets. High protein diet or the ingestion of phenylalanine and phenylpyruvic acid result in an increased daily output of phenyl compounds. Data on sweat excretion of phenyl compounds are also presented.

20. Hier, S. W., Cornbleet, T., and Bergeim, O., *J. Biol. Chem.*, 1946, v166, 327.

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## Oligophrenia Phenylpyruvica. II. Constancy of the Metabolic Error. (18109)

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In phenylpyruvic oligophrenia mental deficiency is accompanied by faulty phenylalanine metabolism. The metabolic error is manifested by the urinary excretion of large amounts of phenylalanine, phenyllactic acid,

and phenylpyruvic acid, and by an elevated phenylalanine concentration in the blood. The genetic analysis strongly suggests that this disease is inherited as an autosomal Mendelian recessive and that; therefore, the mental and metabolic disturbance may be caused by the same gene. The question arises whether both abnormalities, when

\* Aided by a grant from the Rockefeller Foundation.

TABLE I. Concentration of L-phenylalanine (PA) and Phenyllactic Acid (PL) in Serum and Spinal Fluid of Patients with Phenylpyruvic Oligophrenia.

Patient	Age	I.Q.	Sample I		Sample II		Sample III		Sample IV		Spinal fluid PA mg %
			PA + PL mg %	PA mg %	PA + PL mg %	PA mg %	PA + PL mg %	PA mg %	PA + PL mg %	PA mg %	
1	28	5	32	31	39	38	33	33	33	34	7.7
2	42	46	29	29	33	32	31	28	33	30	7.6
3	41	11	27	27		34					
4	32	7	33	32	31	29					
5	29	47	33	35	38	36	34	32			7.8
6	26	7	27	27	33	31					
7	15	35	31	31							7.5
8	17	12	33	33							
9	23	7	30	29	34	34	33	33			7.9
10	30	38	27	27							
11	26	10	26	26							
12	6	14	29	27							
13	5	24	27	26							
14	19	9	24	26	29	31	32	31			8.2
15	29	5	33	29							
16	5	24	25	24							
17	27	40	24	22							6.2
18	8	10	21	19							
19	22	6									6.1
20	20	7									6.3

fully developed, are correlated in their quantitative aspects. It has recently been suggested(1) that a positive correlation exists between the degree of mental deficiency and the metabolic disturbance as measured by the excretion of phenylpyruvic acid. Since the excretion of phenylalanine and its metabolic derivatives may only be secondary to the abnormally high blood concentration of the amino acid, it was considered of interest to determine whether a correlation exists between this value and the degree of mental deficiency.

In this report quantitative data on the phenylalanine concentration of the blood and spinal fluid of phenylketonurics are presented.

**Experimental.** Microbiological determinations of phenylalanine and phenyllactic acid in serum ultrafiltrates and in urine were carried out on 18 phenylketonuric fasting patients as described previously(2). Microbiological determinations of phenylalanine in the spinal fluid were carried out on 9 patients according to the directions of Solomon *et al.*(3).

1. Fölling, A., Mohr, O. L., and Kund, L., Publ. of the Norwegian Academy of Science, Oslo, 1945.

2. Prescott, B. A., Borek, E., Brecher, A., and Waelsch, H., *J. Biol. Chem.*, 1949, v181, 273.

Suitable dilutions were made in each sample in order to bring the concentration of the amino acid within the range of the microbiological method. Urinary phenylpyruvic acid was determined according to the method of Friedeman and Haugen(4).

**Results and discussion.** The concentration of L-phenylalanine alone and of the sum of phenyllactic acid and phenylalanine in blood serum was determined in some of these subjects with phenylpyruvic oligophrenia at intervals of several months. It may be seen (Table I) that the difference between the sum of phenyllactic acid and phenylalanine and the amino acid alone was not significant. It may, therefore, be concluded that only traces, if any, of the hydroxy-acid circulate in blood. Although there was considerable variation of the values taken at different times in the same individuals, it would appear that the intraindividual variations were significantly smaller than those found among individuals ( $P < 0.05$ ). This result would indicate that there was a characteristic blood level of phenylalanine for each individual.

3. Solomon, J. D., Hier, S. W., and Bergeim, O., *J. Biol. Chem.*, 1947, v171, 695.

4. Friedeman, T. E., and Haugen, C. E., *J. Biol. Chem.*, 1943, v147, 415.

## OLIGOPHRENIA PHENYL PYRUVICA II

TABLE II. Blood Serum and Urine Concentration of Phenylalanine (PA), Phenylactic Acid (PL), and Phenylpyruvic Acid (PP), After Intravenous Administration of 1.6 g of L-Phenylalanine.

Patient	Min.	Serum		Urine	
		PA + PL mg %	ml	PA + PL mg %	PP mg %
R	0	34	—	—	—
	20	38	—	—	—
	40	36	—	—	—
	60	38	—	—	—
	120	42	—	—	—
S	0	39	250	120	160
	10	43	55	128	115
	20	40	25	85	73
	35	41	75	58	90
	120	40	155	62	181
B	0	33	—	—	—
	10	36	—	—	—
	30	34	—	—	—
	120	34	—	—	—
Control	0	2.0	100	0.5	0
	10	7.7	75	0.6	0
	20	5.4	60	1.5	0
	30	6.1	25	1.4	0

It is clear that there is no correlation between the phenylalanine level in serum and the mental performance expressed as I.Q. score.

The relative constancy of the phenylalanine level in serum offers an explanation for the findings of a relative constancy of the ratios of urinary phenyl compounds to urinary total nitrogen(5). Since the amount of phenylalanine circulating through the kidney will determine the amount of the amino acid and its metabolic derivatives excreted in the urine, it is not surprising that the ratio of phenyl compounds to total nitrogen excreted should be a relatively constant value.

The phenylalanine content of spinal fluid as of the serum of phenylketonurics did not differ greatly among patients (Table I). The increase of phenylalanine over the normal values of spinal fluid was of an order of magnitude similar to that observed in the blood.

To two fasting phenylketonurics and to one control (also a mental defective) 1.6 g of L-phenylalanine was administered intravenously. As seen in Table II, the phenyl-

alanine disappeared very rapidly from the blood into the tissues of the phenylketonuric and the non-phenylketonuric defectives. It was apparently released slowly from the tissues since the urine samples obtained simultaneously with the blood samples did not show a significant change in the content of phenyl derivatives during the first 2 hours after the administration of phenylalanine. However, preliminary analyses of the urine indicate that the total amount was excreted within 48 hours.

Since the phenylalanine level in the blood of phenylketonurics is a relatively constant value which shows no correlation with the mental state, it is of interest to inquire into the mechanism which determines the blood level of the amino acid in this disease. It is probable that the major portion, if not all, of the phenylalanine ingested with the food, is excreted as such or as one of the derivatives in the urine. Small amounts of an amino acid excreted by glomerular filtration would be reabsorbed within the kidney tubuli. In the case of phenylpyruvic oligophrenia the nonutilization of phenylalanine leads eventually to a blood level beyond the ability of the tubuli to reabsorb it. The critical blood level at which phenylalanine is excreted in the urine is not known but the maximal rate of tubular reabsorption in dogs is not exceeded by an increase of the phenylalanine content of plasma by a factor of 10(6). It has to be assumed that in phenylketonurics the inability to metabolize phenylalanine causes a level of the amino acid in blood and consequently in the glomerular filtrate to be established which overwhelms the tubular reabsorption. Although the cause of the increased phenylalanine level as such is the metabolic error, it appears that its quantitative expression is determined by the reabsorptive power of the kidney tubuli. Whether this function of the tubuli is normal in phenylpyruvic oligophrenia or is modified by the disease cannot be decided at present.

6. Russo, H. F., Wright, L. D., Skeggs, H. R., Tillson, E. K., and Beyer, K. H., PROC. SOC. EXP. BIOL. AND MED., 1947, v65, 215.

**Summary.** The serum level of free phenylalanine and phenyllactic acid was determined in 18 patients suffering from phenylpyruvic oligophrenia. No significant amounts of the hydroxy acid were found. The concentration range of the amino acid was between 19 and 38 mg per 100 ml of serum with larger inter- than intra-individual variations. No correlation exists between the degree of mental deficiency and the concentration of phenyl-

alanine in serum. Phenylalanine concentration in the spinal fluid varied from 6.1 to 8.2 mg/100 cc.

It is suggested that although the increased amount of phenylalanine is caused by the metabolic error, its blood plasma level is determined by the ability of the kidney tubuli to reabsorb the amino acid.

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### Comparison of Influenza B Virus Strains from the 1950 Epidemic with Strains from Earlier Epidemics. (18110)

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Shortly after the discovery of influenza B virus(1,2), antigenic differences between strains of the agent were noted(3,4). Numerous reports have appeared in which serological studies revealed antigenic differences between strains which had been passed in series in the mouse lung or between such strains and strains which had been maintained by passage only in the chick embryo (3-9). In general, new strains have been compared with the Lee strain or with other strains obtained from the same epidemic. It should be emphasized that the Lee strain which was recovered in 1940(1) had been through many mouse lung passages. In the past, when egg strains have been compared

with each other(8), they have tended to show closely similar antigenic patterns. If the so-called adaptation of strains of influenza B virus to the mouse lung is associated with unpredictable alterations in antigenic pattern, as occurs with strains of influenza A virus(10,11), then antigenic differences between mouse lung strains of the former virus might be more apparent than real.

During February and March, 1950, there occurred in the New York area, as elsewhere in various parts of the United States, an epidemic of influenza. From some patients studied in the Hospital of the Rockefeller Institute, influenza B virus was recovered; from others, influenza A virus was obtained. Investigations with acute and convalescent sera from the various patients confirmed these findings; certain patients clearly had influenza B, others had influenza A. Because all strains obtained from the 1950 epidemic in this laboratory were recovered after inoculation of chick embryos and because a number of earlier egg strains of influenza B virus were available, an opportunity was provided to compare various strains recovered since 1945, none of which had ever been passed in the mouse lung. The results of serological studies with these strains indicate that egg strains

1. Francis, T., Jr., *Science*, 1940, v92, 405.
2. Magill, T. P., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, v45, 162.
3. Eaton, M. D., and Beck, M. D., *PROC. SOC. EXP. BIOL. AND MED.*, 1941, v48, 177.
4. Gordon, I., *J. Immunol.*, 1942, v44, 231.
5. Burnet, F. M., Beveridge, W. I. B., and Bull, D. R., *Austr. J. Exp. Biol. and Med. Sci.*, 1944, v22, 9.
6. Francis, T., Jr., Salk, J. E., and Brace, W. M., *J. Am. Med. Assn.*, 1946, v131, 275.
7. Hirst, G. K., Vilches, A., Rogers, O., and Robbins, C. L., *Am. J. Hyg.*, 1947, v45, 96.
8. Hirst, G. K., *J. Exp. Med.*, 1947, v86, 367.
9. Lazarus, A. S., *PROC. SOC. EXP. BIOL. AND MED.*, 1949, v71, 317.

10. Hirst, G. K., *J. Exp. Med.*, 1947, v86, 357.
11. Suggs, J. Y., *J. Bact.*, 1949, v58, 399.

of influenza B virus obtained from various epidemics occurring in different years may show marked variations in antigenic pattern. Such antigenic dissimilarities appear to be as wide as those between egg strains of influenza A virus(12-16).

*Materials and methods. Viruses.* Three strains of influenza B virus recovered in this laboratory from patients in the 1950 New York epidemic were studied: MB(1), 3rd embryo passage, and MB(2), 3rd embryo passage, represent separate recoveries from one patient admitted to this hospital from Brooklyn, N. Y.; IB1, 5th embryo passage, and IB2, 3rd embryo passage, represent separate strains from 2 patients studied at Irvington House, N. Y. The following strains were received from Dr. T. P. Magill, State University Medical Center at New York: Seattle, 5th embryo passage, recovered at Seattle during 1948-1949; Chaddick, 19th embryo passage, recovered at Buckley Field in 1945; Czech, 9th embryo passage, recovered at Camp Edwards in 1945; I-B-45, 14th embryo passage, recovered at Iowa City in 1945. In addition, B1103, 10th embryo passage, recovered in 1947, was obtained from Dr. R. M. Taylor, International Health Division, The Rockefeller Foundation. All strains had been recovered in the chick embryo; none had been passed in the mouse lung. For purposes of comparison the mouse adapted Lee strains as well as the PR8 strain of influenza A virus were included.

*Sera.* Immune hamster sera were obtained 3 weeks after the intranasal inoculation of a  $10^{-2}$  dilution of allantoic fluid infected with the desired strain. In each instance serum was pooled from 10 or more immune animals. Immune rabbit antisera were prepared as described previously(17). In each case a pool

12. Rasmussen, A. F., Stokes, J. C., and Smadel, J. E., *Am. J. Hyg.*, 1948, v47, 142.

13. Kalter, S. S., Chapman, O. D., Feeley, D. A., and MacDowell, S. L., *J. Immunol.*, 1948, v59, 147.

14. Taylor, R. M., *Am. J. Pub. Health*, 1949, v39, 171.

15. Hilleman, M. R., Mason, R. P., and Rogers, N. G., *Pub. Health Rep.*, 1950, v65, 771.

16. Archetti, I., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1950, v92, 441.

of sera from 2 rabbits was employed. Immune ferret and rabbit sera against B1103 were kindly provided by Dr. R. M. Taylor.

*Hemagglutination-inhibition antibody titrations.* A final concentration of 4 hemagglutinating units of virus and 2-fold dilutions of serum were employed in all tests. The procedure and the estimation of the end point were identical to those described previously (17). Rabbit and ferret sera were inactivated in a 1:2 dilution in saline at 65°C for 30 minutes; hamster sera at 56°C for 30 minutes. Rabbit antisera were absorbed with packed chicken erythrocytes resuspended in the sera to give a concentration of 20% at 4°C to remove anti-chick RBC agglutinins. In addition, both rabbit and ferret immune sera were treated to eliminate the so-called nonspecific inhibitor in the following manner: equal volumes of serum and PR8 infected allantoic fluid were mixed, held at 42°C for 3 hours, and then heated at 65°C for 30 minutes to eliminate the virus. With the control anti-PR8 serum a similar procedure using Lee virus was employed. Tests with a number of different viruses showed that this procedure was effective in eliminating almost all of the inhibitor.

*Neutralizing antibody titrations.* A  $10^{-4}$  dilution of allantoic fluid infected with the desired virus and 2-fold dilutions of inactivated serum were employed in all titrations. The method used was identical to that described previously(17). Each mixture was inoculated into a group of 3 chick embryos; the end point was determined on the basis of the occurrence of hemagglutination with allantoic fluids obtained from each of the embryos inoculated.

*Experimental.* Results of cross hemagglutination-inhibiting antibody titrations with immune sera and various egg strains of influenza B virus recovered in different years are shown in Table I. It appears evident that all of the strains studied differed markedly from the mouse-adapted Lee strain. The extent of the dissimilarity between these strains and the Lee strain was nearly as

17. Walker, D. L., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1950, v91, 65.

TABLE I. Cross Hemagglutination-Inhibiting Antibody Titrations with Various Strains of Influenza B Virus.

Virus*	Immune serum			
	MB†	B1103‡	Lee†	PR8†
Serum titer				
MB (1) 1950	128	32	0	0
,, (2)	64		0	0
IB1	64		0	0
IB2	32		0	0
Seattle 1948-49	16	0	0	0
B1103 1947	64	1024	16	
Chaddick 1945	64	128	0	0
Czech	64		16	0
I-B-45	32		16	0
Lee 1940	0	128	512	0
PR8	0	0	0	2048
Rabbit antiserum				
MB (1) 1950	768	.96	96	0
,, (2)	256	64	128	0
IB1	256	64	32	0
IB2	256	64	64	0
Seattle 1948-49	256	32	64	0
B1103 1947	512	1024	512	
Chaddick 1945	512	256	512	0
Czech	512	128	512	0
I-B-45	256	128	512	0
Lee 1940	384	48	6144	0
PR8	0	0	0	4096

\* 4 units final.

† Hamster serum.

‡ Ferret serum.

Lowest serum dilution = 1:8. Rabbit and ferret serum were treated to eliminate inhibitor.

striking when rabbit antiserum was used as when immune hamster or ferret serum was employed. Of more importance is the evidence that there were wide differences in the antigenic composition of various egg strains. This is especially evident when the cross reactions obtained with MB and B1103 as well as those with Seattle are compared. The absence of any reaction between the anti-influenza B virus sera and PR8 provide an indication that the nonspecific inhibitor had been effectively eliminated by the procedure described above.

When the titer ratios, *i.e.*, homologous titer divided by heterologous titer(8), obtained with MB and B1103 are compared, the extent of the antigenic difference between the 2 strains becomes readily apparent. It has been shown(16) that with any 2 strains the degree of antigenic difference can be expressed in a single figure derived from the

2 heterologous titer ratios,  $r_1$  and  $r_2$ , by means of the function:  $r = \sqrt{r_1 \times r_2}$ . With hamster or ferret immune serum,  $r = 1/8$ , indicative of an antigenic cross relation between MB and B1103 of the order of but 12%; with rabbit antisera,  $r = 1/4$ , indicating an antigenic relation of approximately only 25%. Although immune sera against Seattle were not available, the reactions with heterologous sera against MB and B1103 provide a clear indication of the extent of the antigenic dissimilarity among these 3 egg strains. It is apparent that relatively little difference was demonstrated among the three 1945 strains. Similarly, marked indications of differences among the three 1950 strains were not found with available sera. However, that the 1947 strain (B1103) possessed an antigenic composition different to either the 1945 or the 1950 strains seems clear. With Lee and MB the hamster and rabbit sera gave  $r$  ratios of 1/64 and 1/11, respectively, indicating an antigenic relationship of no more than 1 to 9%.

The results of cross neutralizing antibody titrations *in ovo* between MB and mouse adapted Lee are shown in Table II. The titer ratios obtained by the *in vivo* procedure provide adequate confirmation of the results obtained with the *in vitro* technic and indicate that the Lee strain was antigenically dissimilar to MB. Computations showed that with hamster immune sera the  $r$  ratio for these two strains = 1/8 while with rabbit antisera the  $r$  ratio = 1/16, indicating a cross relation between MB and Lee of no more than 6 to 12%.

TABLE II. Cross Neutralizing Antibody Titrations *in ovo* with MB and Lee Strains of Influenza B Virus.

Virus*	Serum titer		
	MB	Lee	PR8
Hamster immune serum†			
MB	16	4	0
Lee	0	32	0
Rabbit antiserum†			
MB	128	16	0
Lee	16	512	0

\* 10<sup>-4</sup> dilution allantoic fluid.

† Lowest serum dilution = 1:4.

*Discussion.* That there are antigenic differences between various strains of influenza B virus has been clear for a considerable time(3-9). Due to the possibility that so-called adaptation to the mouse lung is associated with alterations in the antigenic pattern of strains of influenza B virus, as appears to be the case with influenza A virus(10,11), it has not been clear that the antigenic differences observed reflected dissimilarities in strains as they were obtained from man. The results of the present study indicate that strains of influenza B virus recovered on inoculation of the chick embryo and maintained but for a few passages in this species may be markedly dissimilar in antigenic composition despite the fact that they were not passed in the mouse lung. Results obtained recently in this laboratory(16) indicate that as many as 12 serial passages of influenza A virus strains in the chick embryo cause no demonstrable alteration in antigenic pattern. It seems probable that similar results would be obtained in comparable experiments with influenza B virus.

That the differences observed were most evident with strains of influenza B virus obtained in different years is consistent with findings relative to strains of influenza A virus(12-16). Moreover, the extent of the dissimilarity in antigenic pattern appears to be comparable to that found among egg strains of influenza A virus. Since 1947 many workers have referred to strains of influenza A virus which differed strikingly from earlier strains as "A prime" strains(18). If the mouse adapted Lee strain is taken as the standard of reference for influenza B virus,

as has been done commonly, then it would be consistent with current usage to designate most of the other strains included in this study as influenza B prime strains. This practice seems undesirable and cannot be recommended; it tends to separate strains into artificial antigenic categories and increases markedly the difficulty of classification and relation. Present evidence suggests that among strains identifiable as influenza B virus there is a more or less continuous spectrum of antigenic differences. At this time it appears impossible to define antigenic categories with satisfactory precision.

The bearing of these findings upon the problem of artificial immunization of man against influenza B requires some comment. It may be that the differences found are not sufficiently great to affect significantly the immunity induced with a vaccine prepared with the Lee strain. Evidence obtained in 1945(6) suggested that this was the case. However, strains recovered in 1950 as well as in 1948-1949(9) appeared to be even more dissimilar to Lee than were those obtained in 1945. It seems possible that current strains are sufficiently different to Lee to justify some doubt as to the desirability of continuing to use the Lee strain exclusively for vaccine production.

*Summary.* Cross serological reactions indicated that egg strains of influenza B virus obtained in different years were dissimilar in antigenic composition. The extent of the differences in antigenic pattern was analyzed. All strains studied were antigenically different from the mouse adapted Lee strain. The implication of these findings on the problem of immunization in man was discussed.

18. Salk, J. E., and Suriano, P. C., *Am. J. Pub. Health*, 1949, v39, 345.

## Effect of Length of a Ryanodine-treated Muscle on Oxygen Uptake. (18111)

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In muscle physiology there are many factors which are related to the length of the muscle. To mention just a few, there are: birefringence(1), maintenance heat(2), shortening heat(3), tension(4), and oxygen consumption of a resting muscle after stretching (5,6). Evidence is given in this paper to show that the length also plays an important part in energy release in the muscle treated with Ryanodine even though excitation and mechanical work have been reduced to a minimum.

**Methods.** Frog sartorius muscles were dissected out and allowed to equilibrate in Ringer's-phosphate solution for 18 to 24 hours at 4°C. Ryanodine, in a final concentration of  $1 \times 10^{-5}$ , was introduced under two different conditions. In the first case, Ryanodine was introduced from a side-arm of a Warburg flask in the usual manner. The Ryanodine was dumped into the solution containing the muscle after a normal period of one hour which had followed an appropriate equilibration period of 20 minutes.

The second method of introduction of the Ryanodine was designed to allow for a more even distribution of the drug throughout the muscle. This was accomplished by cooling to 4°C the Warburg flasks containing the muscle and Ringer's as well as the Ryanodine solution before dumping the drug. A 30-minute interval was allowed for diffusion

of the drug through the muscle before the temperature was raised. The action of Ryanodine resumed its normal course after it was brought back to the experimental temperature (23°C). More regular and more even contractures were produced by this procedure.

In the later experiments the muscle length was measured by locating the ends of the muscle with calipers and measuring the distance between the points on a millimeter scale. The rest length of the muscle was taken by measuring the length of the muscle in the animal when the leg was extended.

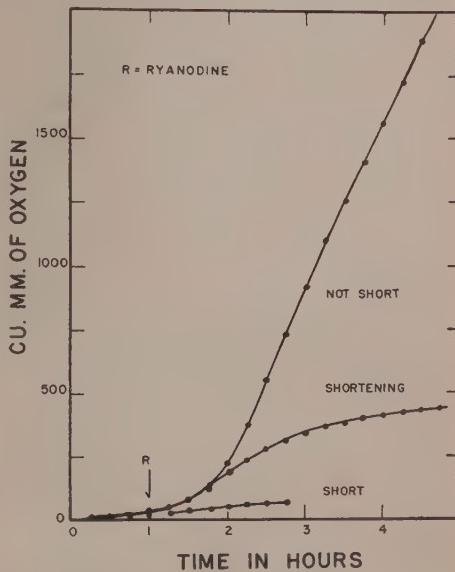


FIG. 1.

Comparison of total oxygen consumption of shortened and unshortened Ryanodine-treated frog muscle. Final concentration of Ryanodine in all 3 curves was one part in 100,000. The top curve represents 8 experiments in which the muscles had not shortened noticeably at the end of the experiment. The middle curve represents 9 experiments in which the muscles had shortened markedly by the end of the experiment. In the curve marked "short," the muscles in 8 experiments were allowed to shorten first and then the oxygen consumption was measured for the period indicated. The arrow marked "R" does not apply for this curve.

\* This work was done under contract between the Office of Naval Research and the Medical College of Virginia.

1. Fischer, E., *J. Cell. and Comp. Physiol.*, 1938, v12, 85.
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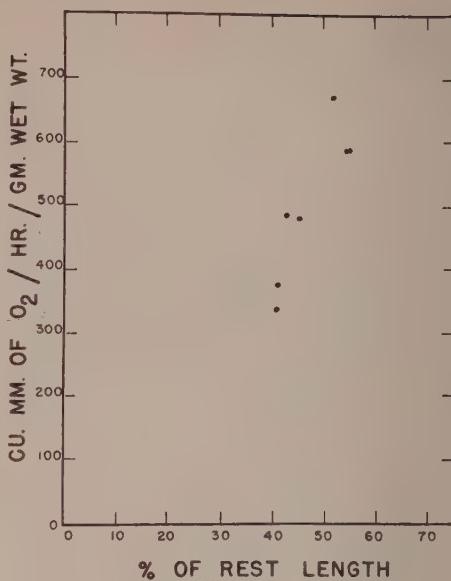
Shortening was calculated as per cent of rest length.

*Results.* During a study of the action of inhibitors on Ryanodine-treated muscles it was observed that the shortened muscles had a lower oxygen consumption than did the longer ones, regardless of the inhibitors used. In the first figure the total oxygen consumption of the muscle has been plotted against time. The 3 curves represent averaged data from 3 different groups of experiments. The first curve represents the average oxygen consumption of 8 experiments in which the muscles had not shortened noticeably. In the second curve are the averaged results from 9 experiments including all the muscles which had shortened and were classed as markedly shortened at the conclusion of the experiment. In both these groups Ryanodine alone had been introduced from the side arm after a normal resting level had been established for one hour. In the third curve, representing the averaged results of eight experiments, the muscles were allowed to shorten first and then the oxygen level was measured. These curves clearly indicate that there is a marked difference between the oxygen consumption of the unshortened muscles as compared to the contracted muscles.

In the second figure is given the oxygen consumption of one muscle as it gradually contracted. The measurements of length were made by observing the muscle through the glass flask and are therefore somewhat inaccurate. Even so, this curve indicates that there is a gradual decrease in oxygen consumption as the muscle shortens. Most muscles do not shorten this slowly under the influence of Ryanodine so this type of analysis can not always be made.

In the last phase of this work (Fig. 3), an attempt has been made to correlate the oxygen consumption with the length of the muscle. The length reported was the length of the muscle as measured at the end of the experiment. Thus each point in this figure represents one complete experiment.

In addition the length of the muscle in about half of the experiments was estimated through the flask after every reading of the manometer. These observations were useful



Effect of length on oxygen consumption as measured by one muscle. The final concentration of Ryanodine was one part in 100,000. The length was measured in the flask as the oxygen consumption was measured.

in determining when most of the shortening had taken place; however, they are not included in the graph.

In general the shortening of the muscle takes place very rapidly once it starts. The oxygen consumption was taken over that part of the oxygen curve where the oxygen requirement had leveled off, immediately after the muscle had stopped shortening. In order to determine this value, the rate of oxygen consumption was plotted against time for each determination. These curves are not shown in the text.

In those experiments in which the lengths were not estimated after each reading of the oxygen uptake, the final oxygen uptake for a given length was taken after the curve had leveled off, following the initial oxygen spurt at the beginning of the contracture. This oxygen consumption was the average for this level portion of the curve and represents several oxygen measurements.

For each muscle length down to the 30% of rest length, there is a corresponding oxygen consumption when the muscles have been

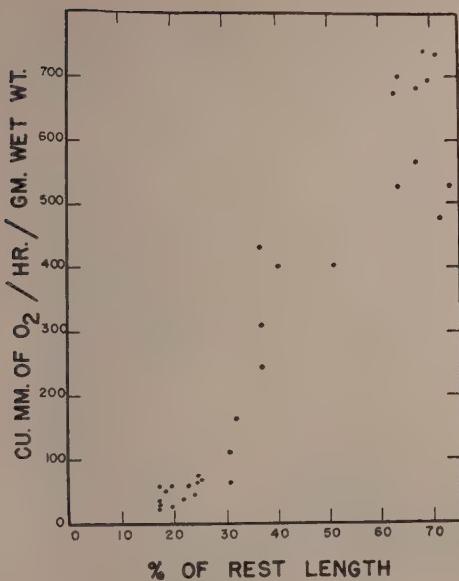


FIG. 3.

Effect of length on oxygen consumption as measured by separate muscle experiments. The length of the muscle was measured at the end of the experiment using calipers. One part in 100,000 was the final concentration of Ryanodine.

treated with the same concentration of Ryanodine. Below 30% of the rest length the oxygen consumption has fallen to at least the resting metabolism level and remains at this level or less with still shorter muscles.

**Discussion.** The action of Ryanodine is an interesting one. This drug releases a large amount of energy in the muscle even though the muscle is unable to do any appreciable mechanical work or to be stimulated electrically. G. A. Edwards and co-workers(7) have demonstrated that the excitation process is completely blocked by Ryanodine. In repeating part of this work, we found that the blocking of excitation occurred well before the contracture developed. In the present work a much higher increase in the oxygen consumption of frog muscle was found with Ryanodine-treated muscles than was reported by Edwards and co-workers(7).

In those instances in which the drug was allowed to diffuse evenly into the muscle at

low temperature before being brought to room temperature the muscle fibers in most cases contracted in perfect alignment if allowed to shorten under no load. However, if any restraining force were used, compelling the muscle to develop tension as it shortened, then considerable damage was done to the fibers.

Many investigators have worked on contractures. Contractures in general have at least one advantage over the simple twitch in the study of muscle physiology. The contracture develops over a longer period of time than a simple twitch and thus allows for a measurement of energy as well as for oxygen consumption during the period of shortening.

Since under the conditions in which Ryanodine failed to cause a marked shortening or contracture the oxygen consumption remained extraordinarily high and only diminished with contracture or shortening, it is believed that it is the shortening and not the drug which causes the final lowering of the oxygen consumption. It thus appears that the length of the muscle regulates any excess oxygen consumption above the resting level. Other contractures probably act in a similar manner to that of the Ryanodine-produced contracture. For example, caffeine increases the oxygen consumption of muscle to almost the same magnitude as does Ryanodine. In addition, a higher concentration of caffeine produces contracture. Thus contracture and sub-contracture levels are mentioned in the literature. More consistently higher oxygen uptakes are found with concentrations slightly below the contracture level than above it(8). The authors of this present investigation would like to suggest that this mechanism (produced by Ryanodine) exists also in other forms of contracture.

As judged by the oxygen consumption shown in Fig. 3, it can be deduced that the length of the muscle controls the release of energy within the muscle. It appears that the energy is first released in certain fixed amounts, depending upon the stimulating condition or drug and then the energy is further

7. Edwards, G. A., Weiant, E. A., Slocombe, A. G., and Roeder, K. D., *Science*, 1948, v108, 330.

8. Fenn, W. O., *J. Pharmacol. and Exper. Therap.* 1931, v62, 81.

controlled by the muscle length. More exact methods of recording length and oxygen consumption simultaneously will be devised to improve the accuracy of the curve showing this relationship. This curve should also be very useful in determining other theoretical energy calculations involved in muscle physiology.

**Conclusions.** Ryanodine causes a high oxygen consumption in muscles which do not shorten. In those muscles that shorten the oxygen consumption falls off as the muscle shortens. The length of the muscle seems

to regulate the oxygen consumption of the Ryanodine-treated muscle. Complete cutting off of the extra metabolism due to Ryanodine occurs at 30% of the rest length.

We are indebted to Dr. Roeder and to Merck and Co. for supplying us with the purified Ryanodine powder.

We wish to thank Dr. Robert W. Ramsey for his helpful advice and interest in this problem. We are also indebted to him for making many of the microscopic observations on the structure of the muscle at the conclusion of the experiments.

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## Pharmacology of Para-substituted Derivatives of Diphenhydramine. (18112)

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Among a number of the derivatives of 2-benzhydryloxy-N,N-dimethylethylamine (diphenhydramine), tested for their antihistaminic activity, the para-substituted compounds were found of special interest from the standpoint of the relationship between chemical constitution and pharmacological action. In this report, a comparison of the pharmacological properties will be made of the para-alkyl, para-methoxy and the para-halogen-derivatives of 2-benzhydryloxy-N, N-dimethylethylamine with a view of appraising their therapeutic applicability.

**Materials and methods.** The following compounds,\* p-methyl, p-ethyl, p-n-propyl, p-methoxy, p-fluoro, p-chloro, p-bromo and p-iodo benzhydryloxy-N, N-dimethylethylamine as their hydrochlorides, have been investigated. They are white crystalline compounds, freely soluble in water.

The antihistamine potency of a compound was determined: (a) by protection against the lethal toxicity of histamine aerosol in guinea pigs, and (b) by suppression of the histamine-induced contraction of an isolated intestinal strip with procedures as previously

described(1,2). Similar technics were employed for estimating anticholinergic and myotropic spasmolytic activities. Acetyl-beta-methylcholine chloride (methacholine) was used to produce a fatal cholinergic bronchospasm to guinea pigs(3). At least 48 animals, 12 per group for each dose, were used to determine a 50% protective dose of the drug.

Acute toxicity was determined in mice by intraperitoneal injection. The LD<sub>50</sub> and average standard error were calculated from the mortalities, obtained with 5 doses of the drug in groups of 20 animals each, by the method of Miller and Tainter(4).

The potentiation of epinephrine by and the atropine-like action of these compounds on blood pressure were investigated in dogs under pentobarbital anesthesia with standard procedures.

**Results and discussion.** As the data in

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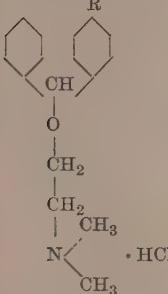
2. Chen, G., Ensor, C. R., and Clarke, I., *J. Pharm. Exp. Therap.*, 1948, v92, 90.

3. Chen, G., and Ensor, C. R., *J. Lab. Clin. Med.*, 1949, v34, 1010.

4. Miller, L. C., and Tainter, M. L., *PROC. Soc. EXP. BIOL. AND MED.*, 1944, v57, 261.

\* Kindly made available to us by Drs. R. W. Fleming and G. Rieveschl, Chemical Division Research Laboratories, Parke, Davis and Co.

TABLE I. Efficacy of p-Substituted Diphenhydramine Derivatives in Preventing Fatal Histamine-induced and Mecholyl-induced Bronchoconstriction in Guinea Pigs; and Acute Toxicity in Mice.

Compound	Antihistamine			Toxicity		Therapeutic index†	Anti-cholinergic mg/kg (P.D. 50, I.P.)
	mg/kg P.D. 50 ± S.E. (I.P.)	Activity ratio*	L.D. 50 ± S.E. (I.P.)	Activity ratio	Toxicity ratio		
							
R = H†	1		56 ± 2.2	1	1	25.0	
R = CH <sub>3</sub> (R = H)	0.73 ± 0.14§ (2.08 ± 0.44)	2.85 ± 0.82 S.E.	150 ± 2.0	.37	7.7	Ineffective at 50	
R = C <sub>2</sub> H <sub>5</sub>	2		117 ± 4.0	.48	4.2	Ineffective at 50	
R = n-C <sub>3</sub> H <sub>7</sub>	1		67 ± 1.6	.84	1.2		
R = CH <sub>3</sub> O	1		148 ± 2.4	.38	2.6	Ineffective at 50	
R = F	3		57 ± 2.0	.98	3.1	"	
R = Cl	1		79 ± 2.7	.71	1.4	"	
R = Br (R = H)	1.25 ± 0.20   (2.70 ± 0.67)	2.16 ± 0.64 S.E.	108 ± 2.5	.50	4.3	"	
R = I	3		92 ± 3.3	.61	4.9	"	

\* The activity of each compound was determined simultaneously with that of diphenhydramine with the same stock of guinea pigs.

† Diphenhydramine.

‡ Assuming that the toxicity in guinea pigs is directly proportional to that in mice.

§ 7 experiments.

|| 3 experiments.

Table I indicate, para-substitution with a halogen or an alkyl group results either in an enhancement of antihistamine activity, a decrease in toxicity or both. The anticholinergic action, on the other hand, is greatly diminished by the substitution. In the alkyl-substituted compounds, the methyl group is the most effective giving a therapeutic index seven times that of the parent substance by an increase in activity and a decrease in toxicity. The lengthening of the alkyl chain decreases the therapeutic efficacy. In the halogen series, with the exception of fluorine, the therapeutic index increases with the atomic

weight. By substitution with fluorine, the antihistamine activity is increased, while the toxicity remains about the same. The para-methoxy derivative of diphenhydramine is equal to the parent compound in activity but only half as toxic.

The *in vitro* results in Table II, obtained with the isolated ileal strip, are generally in agreement with those from histamine aerosol and from mecholyl aerosol in guinea pigs. The myotropic spasmolytic effect of the para-substituted compounds, as determined by suppression of BaCl<sub>2</sub>-induced contraction of the gut, is approximately of the same magnitude

TABLE II. Concentrations of p-Substituted Diphenhydramine Derivatives Which Inhibit the Spasmogenic Effects of Histamine, Acetylcholine, and Barium Chloride on Isolated Guinea Pig Ileum.

Compound	Histamine activity* ratio		Acetylcholine activity* ratio		Barium chloride activity* ratio	
R = H	.0058 γ/cc†	1	.350 γ/cc†	1	2.55 γ/cc†	1
R = CH <sub>3</sub>	.0028 "	2.07	2.175 "	0.16	2.56 "	1.04
R = H	.0066 "	1	.125 "	1	1.51 "	1
R = C <sub>2</sub> H <sub>5</sub>	.0082 "	0.80	2.500 "	0.05	1.40 "	1.08
R = H	.0138 "	1	.0122 "	1	1.47 "	1
R = n-C <sub>3</sub> H <sub>7</sub>	.0338 "	0.41	.738 "	0.17	0.99 "	1.49
R = H	.0072 "	1	.0995 "	1	1.47 "	1
R = CH <sub>3</sub> O	.0107 "	0.76	1.8965 "	0.05	1.79 "	0.82
R = H	.0131 "	1	.0955 "	1	0.25 "	1
R = F	.0073 "	1.79	.1966 "	0.48	0.38 "	0.66
R = H	.0218 "	1	.0547 "	1	3.95 "	1
R = Cl	.0140 "	1.56	.1986 "	0.27	2.30 "	1.70
R = H	.0101 "	1	.0775 "	1	0.97 "	1
R = Br	.0074 "	1.36	.3150 "	0.24	1.09 "	0.89
R = H	.0127 "	1	.1277 "	1	4.06 "	1
R = I	.0088 "	1.45	.8210 "	0.16	2.38 "	1.62

\* The activities of diphenhydramine and the derivative being compared on the same intestinal strip.

† Concentration that inhibits 50% of the maximal contractions by the spasmogenic agent, each value representing an average of 2 determinations on different strips.

as that of diphenhydramine.

The modification of antihistamine potency and toxicity of parasubstitution has been reported for tripelennamine(5). In tripelennamine, para-substitution with a methoxy group (Neoantergan) increases the antihistaminic activity with no change in toxicity. The para-bromobenzyl derivative is less toxic but not more active than the parent compound. On the other hand, the antihistaminic activity is somewhat lowered by para-substitution with a methyl group, and activity is almost lost in the ethyl analog. In contrast, an increase of antihistaminic activity results by adding a

halogen, methyl or an ethyl radical at the para-position of diphenhydramine.

Like diphenhydramine, the para-substituted derivatives produced a slight rise in blood pressure in the dog with small intravenous doses and an immediate transient fall following large doses. They potentiate the vasopressor effect of epinephrine and antagonize the vasodepressor effect of acetylcholine. Their atropine-like action, however, is much less than that of diphenhydramine.

The manifestations of acute toxicity in mice of these p-substituted compounds are qualitatively similar to those of diphenhydramine, viz., excitement followed by depression.

The therapeutic efficacy of the para-methyl

and para-bromo derivatives of diphenhydramine has been appraised clinically. The collective data indicate that their antihistaminic activity is comparable to that of diphenhydramine. The incidence of drowsiness and atropine-like effects is low with the para-methyl derivative(6,7); it is insignificant with the para-bromo compound(8).

6. Schulman, P. M., and Fuchs, A. M., *Ann. Allergy*, 1949, v7, 502.

7. McGavack, T. H., et al., *J. Allergy*, 1950, v21, 353.

8. Sharp, E. A., personal communication.

**Summary.** The antihistaminic, anticholinergic and musculotropic spasmolytic properties of the para-halogen, para-methoxy and some para-alkyl derivatives of 2-benzhydryloxydimethylethyl amine have been investigated. Para-substitution with methyl, ethyl and halogen atom results in an enhancement of antihistaminic activity, a decrease in acute toxicity, or both. It lowers the atropine-like action but does not produce a significant change in musculotropic spasmolytic activity.

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## Hypoadrenalinism: Steroidal Mediation of Sodium Action on Blood Pressure; Modification of Antiarthritic Response to Cortisone.\* (18113)

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Considerable evidence has accumulated that both the sodium ion and the adrenal cortex are concerned to some extent with the maintenance of blood pressure levels in hypertensive patients(1-3). Although the rigid restriction of salt masks the pressor response of hypertensives to desoxycorticosterone(4), this type of observation does not establish the fact that alterations in sodium metabolism modify the arterial tension through an adrenal mechanism. It has been noted that the adrenals of rats on a reduced sodium intake may be smaller and different in color(5),

temporarily depleted of ascorbic acid(6), and may show secretory changes(7) and (in nephritic animals) subcapsular hyperplasia(8). Furthermore, excessive salt, or liberal amounts in animals receiving desoxycorticosterone, has resulted in progressive structural and functional atrophy of the glomerular zone of the adrenal cortex(9,10). Finally, chromatographic patterns of urinary steroid excretion may be influenced by the salt intake(11).

In order to investigate further the possibility that sodium influences the blood pressure through steroid action, studies were

\* This study was supported in part by a research grant from the National Heart Institute, U.S.P.H.S., and was aided through the generosity of the Albert and Mary Lasker Foundation, the Albert H. and Jessie D. Wiggan Foundation, and the Masonic Foundation for Medical Research and Human Welfare.

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6. Danford, H. G., and Herrin, R. C., *Am. J. Physiol.*, 1949, v159, 566 (abstract).

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11. Genest, J., Cotzias, G. C., Dahl, L. K., Eisenmenger, W., and Dole, V. P., *Fed. Proc.*, 1950, v9, 175.

undertaken in a patient with uncomplicated hypertensive vascular disease and Addison's disease who also had mild diabetes mellitus and rheumatoid arthritis.

*Case report and methods.* G.S., a 55-year-old, white housewife, was admitted to the metabolism ward of the Presbyterian Hospital for research study. Hypertension was first recorded 10 years before admission, with repeatedly elevated blood pressure readings up to 210/110 mm of mercury. Addison's disease developed one year before entry, as demonstrated by weakness, skin and buccal mucous membrane pigmentation and the disappearance of hypertension. The diagnosis was clinically apparent and was substantiated by the finding of repeated serum sodium values which were markedly below normal limits following salt withdrawal, calcification in the adrenal areas by x-ray and abnormally low 17-ketosteroid (2.9 mg) and neutral reducing lipid values (0.9 mg)(12)<sup>†</sup> in 24-hour urine specimens. The administration of 50 mg of adrenocorticotrophic hormone (Armour) failed to produce any lowering of the eosinophile count. Mild diabetes was first evident 7 years before, as manifested by hyperglycemia, glycosuria and decreased tolerance to glucose, but was adequately controlled by diet alone. The diagnosis of rheumatoid arthritis, although not substantiated serologically, was made on the basis of 10 years of characteristic distribution of joint pains and deformities, the typical pattern of stiffness after inactivity, and the absence of positive data favoring other types of arthritis. There was neither past nor present evidence of cardiac pain, congestive failure, renal or cerebral involvement. Funduscopic examination showed minimal arteriolar narrowing. Complete blood count and venous pressure were within normal limits. Popliteal arterial pressures were comparable to those obtained in the arms. X-ray of the heart disclosed no hypertrophy and the electrocardiogram showed no abnormalities. Repeated urinalyses were negative. The patient had no

12. Heard, R. D. H., Sobel, H., and Venning, E. H., *J. Biol. Chem.*, 1946, v165, 687 and 699.

<sup>†</sup> We are indebted to Dr. Joseph W. Jailer for these determinations.

significant temperature elevation throughout the period of observation, and at no time showed signs or symptoms of congestive failure.

The methods employed were identical to those previously described(4,13), including the measurement of the "resting" blood pressure, except that the patient was allowed water *ad libitum* for the first 94 days always in excess of 2000 cc. Thereafter the fluid intake was kept constant at 4550 cc daily. She was provided with a constant diet containing 50 g of protein and 225 g of carbohydrate daily. A large salt intake was found obligatory to sustain normal serum sodium values. Although a constant sodium chloride intake could not be maintained exactly, interval chemical analyses showed that the intake ranged between 290 and 350 milliequivalents per day. Supplemental salt was provided in the form of enteric-coated tablets. Desoxycorticosterone acetate<sup>‡</sup> (DCA) was administered subcutaneously in single daily doses, and cortisone<sup>§</sup> intramuscularly in 3 divided doses per day.

*Results.* (Fig. 1) The patient was placed initially on a regimen of salt restriction (A). Hypoadrenalinism was confirmed again by the appearance of nausea and increased weakness and a drop in serum sodium concentration to 128 milliequivalents per liter. Thereafter, by avoiding restriction of salt, serum sodium values were never permitted to fall below normal limits and she remained free of manifestations of adrenal cortical insufficiency.

The addition of 25 mg of cortisone daily (B) was accompanied by subjective improvement in the arthritis, definite objective increase in joint mobility and a progressive reduction in the erythrocyte sedimentation rate. After restoration of electrolyte equilibrium by the resumption of adequate salt, the administration of cortisone was associated with no other subjective changes, no further significant effects on salt and water balance, no gly-

13. Perera, G. A., Pines, K. L., Hamilton, H. B., and Vislocky, K., *Am. J. Med.*, 1949, v7, 56.

<sup>‡</sup> Supplied through the generosity of Dr. K. W. Thompson of Organon, Inc., Nutley, N. J.

<sup>§</sup> Purchased from Merck and Co., from funds supplied by the U.S.P.H.S.

G.S. #55 #987388.

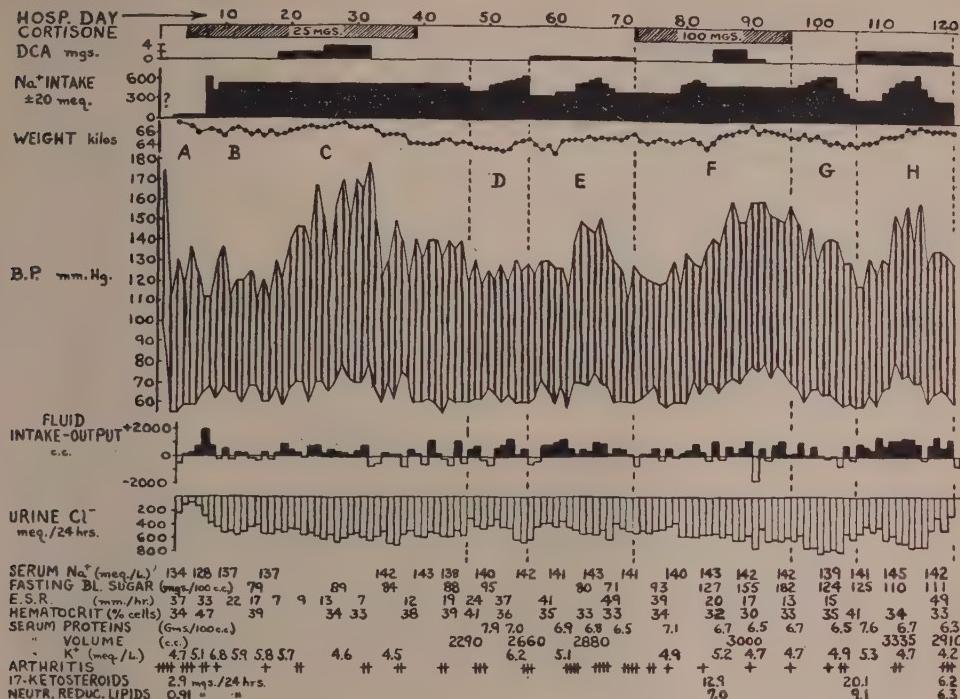


FIG. 1.  
Summary of clinical and laboratory data.

cosuria and no change in fasting blood sugar levels. The "resting" blood pressure was unaffected. On the further addition of DCA (C), first 2 and then 4 mg daily, some hemodilution was observed and a rise in arterial tension was noted. The arthritis remained improved. Prompt diuresis and a rapid fall in blood pressure ensued after stopping the DCA but while the patient was still receiving cortisone. On discontinuing the latter steroid, the arthritis recurred slowly and the sedimentation rate increased.

A progressive increase in sodium chloride intake was instituted next (D). A peak ingestion of close to 40 g of salt was achieved without significant gastrointestinal symptoms. This was accompanied by some weight gain and hemodilution but no alteration in blood pressure. Simultaneously with reduction in the sodium chloride intake, the patient was placed on 1 mg of DCA daily (E). Again the

salt dosage was increased, again hemodilution was evident, but this time with concomitant elevation of the arterial tension. On decreasing the sodium chloride intake, despite continuation of the steroid, the blood pressure fell promptly.

During the next 24 days (F) the patient received 100 mg of cortisone daily in divided doses with almost complete remission of her arthritic symptoms and a progressive decline in sedimentation rate. Slight rounding of the face, insomnia, weight gain, hemodilution and an increase in fasting blood sugar levels developed during this period. Presumably because of a high renal threshold, only inconstant minimal traces of reducing substances appeared in the urine. The 24-hour excretion of 17-ketosteroids rose to 12.9 mg, of neutral reducing lipids to 7.0 mg, after 11 days of cortisone administration alone. The blood pressure began to rise slightly before

the addition of DCA, apparently unrelated to changes in sodium chloride intake, continued its upward trend during 8 days of DCA therapy and did not show a significant fall until the cortisone was withdrawn.

Duplication of the type of study conducted in periods (D) and (E) was then undertaken. The blood pressure dropped progressively on discontinuing cortisone even though the sodium chloride dosage was increased to a peak of 42 g per day for a three-day period (G). With reduction in the sodium chloride intake, the patient was placed on 4 mg of DCA daily (H). Once more the "resting" blood pressure rose with increased salt dosage and fell promptly when it was decreased.

Casual blood pressure values were also recorded throughout the study. During all periods in which a pressor response was noted, casual readings reached definite hypertensive levels with diastolic values in the 90-110 range.

**Discussion.** The response of the arthritis deserves comment first. Although observations were limited to a single study, it is noteworthy that joint signs and symptoms were improved dramatically following a much smaller dose of cortisone than that generally effective in rheumatoid arthritis, and that 100 mg daily were of no greater value than 25 mg. This raises the possibility that the customary action of cortisone in rheumatoid arthritis is offset in part by other substances in the intact adrenal cortex or that its effect is augmented by the hypoadrenal state. There was no indication that the addition of DCA modified the response of the joint disease to cortisone in any respect, or, in the dosages employed, resulted in a paradoxical increase in sodium and chloride excretion(14).

The present study confirms the previously reported finding in a patient with hypertension and hypoadrenalinism, in whom restoration of an elevated blood pressure could be achieved by DCA but not with salt alone(15). In addition, the rise in arterial tension, while the patient was maintained on 100 mg of

cortisone, preceded the addition of DCA and persisted after the DCA was discontinued. This response supports the observation that cortisone may act as a pressor agent in the absence of the adrenals(13).

Finally, it was noted on 2 separate occasions that a high sodium chloride intake without steroid administration failed to modify the blood pressure. On the other hand, when maintained on either 1 or 4 mg of DCA daily, the blood pressure rose with an increased intake and fell when the salt dosage was reduced. The rise in blood pressure might be attributed to the greater hemodilution evident during the periods of DCA administration. Several arguments may be raised against such an explanation. The serum volume was only slightly greater in the first comparative period when receiving DCA. In the final period, the blood pressure fell abruptly to baseline levels on reducing the sodium chloride intake but the serum volume (2910 cc) remained at the levels achieved during the first trial with DCA and salt (2880 cc). Furthermore, we have noted repeatedly that over-treatment with salt and DCA in Addisonian patients may cause comparable degrees of hemodilution; however, hypertension in this group, if it is to appear, requires at least a week or two for its development and occurs long after maximal salt and water retention.

The failure of the blood pressure to rise with sodium chloride doses in excess of 40 g might reflect the presence of some unmeasurable vascular insufficiency or lack of responsiveness in the Addisonian patient. Yet this patient was in fluid and electrolyte equilibrium and salt in these amounts does not modify the blood pressure of normotensives.

The observation in this patient that the level of the blood pressure parallels the sodium chloride intake, providing a steroid source is available, suggests that the action of sodium is mediated through an adrenal mechanism rather than through direct vasoconstrictor action or by means of some independent renal pressor substance. Obviously, this relationship could be at any level of steroid metabolism, before or after degradation, or even through some change in excretion of final end-products

14. Forsham, P. H., Flink, E., Emerson, K., Jr., and Thorn, G. W., *J. Clin. Invest.*, 1949, v28, 781.

15. Perera, G. A., *J.A.M.A.*, 1945, v129, 537

by modification of renal function.

**Summary.** 1. Studies were undertaken in a patient with hypertensive vascular disease, Addison's disease, diabetes mellitus and rheumatoid arthritis. 2. The arthritis responded to smaller doses of cortisone than those generally effective. 3. Whereas large doses of sodium chloride failed to modify the blood pressure,

the arterial tension rose and fell in conjunction with salt intake providing the patient was maintained on constant amounts of desoxycorticosterone acetate. 4. It is suggested that the action of sodium chloride on blood pressure is mediated through an adrenal mechanism.

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## Use of Monosodium Glutamate for Improving the Palatability of Amino Acid Rations.\* (18114)

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It has been observed repeatedly in this laboratory that mice and rats require several days to become accustomed to rations containing free amino acids as the source of nitrogen when fed *ad libitum*. During this adjustment period the animals consume less food and hence grow at a slower rate as compared with those which receive rations containing casein. In some instances a loss in weight occurs during the first 3 to 7 days. Maddy and Elvehjem(1) found that in spite of this lag in growth during the first week, mice that received a ration containing 16 amino acids exhibited growth rates during the second and third weeks that closely approximated those of animals which received a 19% casein ration. Ramasarma *et al.*(2) obtained similar results when rats were fed a ration containing 18 amino acids. Food consumption was variable and lower, and growth rates remained below those of the casein controls until the

third week at which time considerable improvement was noted. The latter workers resorted to the force-feeding and paired-feeding techniques to insure equal nitrogen intakes. Over a period of 2 years we have obtained growth data on 200 mice representing 22 groups that have received *ad libitum* the amino acid ration of Maddy and Elvehjem. The final average weights of these groups ranged between 77 and 98% of those of the casein controls with most of the values being between 85 and 90%. The initial lag period was less pronounced in some groups than others, but it occurred without exception. These concordant findings indicate that a ration of amino acids is not as palatable to the mouse as is a casein ration. It appeared that growth during the first few days of the experimental period was critical in that animals which received the amino acid rations could not overcome completely the initial setback, and final weights rarely completely reached those of animals fed casein.

Since the techniques of force-feeding and paired-feeding are tedious when applied to large groups of animals or are objectionable for other reasons, attempts were made to improve the palatability of the amino acid ration. This report is concerned with the use of monosodium glutamate for this purpose.

**Experimental.** All mice used in these studies were males of the Webster Swiss strain and

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1. Maddy, K. H., Elvehjem, C. A., *J. Biol. Chem.*, 1949, v177, 577.

2. Ramasarma, G. B., Henderson, L. M., and Elvehjem, C. A., *J. Nutrition*, 1949, v38, 177.

were from our stock colony. When 3-4 weeks old, the animals were divided into groups as equally as possible on the basis of weight so that the average weights of the groups within a series differed from each other by not more than 0.3 g. The mice were housed in individual screen-bottom cages and were fed ration and water *ad libitum*. Weighings were made twice weekly during the 21-day experimental period. The amino acid ration fed (hereafter referred to as AAM-2) was the one reported by Maddy and Elvehjem (*loc. cit.*) to be the best for the growth of young mice. This ration contained sucrose, corn oil, salts, vitamins and 16 purified amino acids including 5.03% of L-glutamic acid. In the casein control ration the amino acids were replaced by 19% casein. The nitrogen content of each ration was 2.5%. Monosodium glutamate (MSG), when used, was added at the expense of sucrose, and the amount of L-glutamic acid or casein was altered to maintain the nitrogen content at 2.5%. The average weight gain of the mice receiving the casein ration was assigned a value of 100% in each series, and the final growth of each group receiving an amino acid ration was calculated as a percentage of the growth of the casein group. These data are summarized in Table I.

**Results.** In Series 1 it is seen that although growth on the AAM-2 ration was 98% of that obtained with 19% casein, the addition of 0.5% of MSG resulted in still better growth of 109%. Moreover, the growth curves of this series showed that the group receiving MSG grew at a considerably better rate during the early days of the growth period. In Series 2 the AAM-2 group grew only 86% as much as the casein group. The growth of the groups receiving 0.5% and 1% MSG was 102% and 103% respectively. Growth curves revealed that the 0.5% MSG group surpassed the casein group in rate of growth during the first 3 days, and the 1% MSG group grew at almost the same rate as the casein group during this time. The un-supplemented AAM-2 group lagged in growth over the same period. A further supplement of 2% MSG was tested in Series 3 in addition to the 0.5% and 1% levels. Again in this series the growth of the

TABLE I. Growth Data on Mice Receiving AAM-2 and AAM-2 + MSG.

Series No.	Diet	No. of mice	Avg wt gain (21 days) g	% growth on casein
1	19% casein	10	8.8	100
	AAM-2	10	8.6	98
	AAM-2 + 0.5% MSG	9	9.6	109
2	19% casein	10	8.8	100
	AAM-2	10	7.6	86
	AAM-2 + 0.5% MSG	10	9.0	102
	AAM-2 + 1.0% MSG	10	9.1	103
3	19% casein	11	16.0	100
	AAM-2	11	14.6	91
	19% casein + 1% MSG	11	16.5	103
	AAM-2 + 0.5% MSG	10	14.4	90
	AAM-2 + 1.0% MSG	10	14.1	88
	AAM-2 + 2.0% MSG	11	14.5	91
4	19% casein	22	10.0	100
	AAM-2	22	8.5	85
	AAM-2 + 1.0% MSG	24	9.3	93

AAM-2 group was quite good, 91%, and the addition of MSG produced no significant differences in final growth. However, the group that received the 1% MSG exhibited a better growth rate during the first 10 days than did the AAM-2 group. When 1% MSG was added isonitrogenously to the casein ration, somewhat better growth occurred (103%), and food consumption during the first 10 days was 7% above that of the un-supplemented group.

In Series 4 larger groups of animals were used to determine more accurately the effect of the MSG supplementation. It was found that the growth of the group receiving 1% MSG was 93% as compared with only 85% for the un-supplemented group. Furthermore, the growth rate of the MSG group actually surpassed that of the casein group during the first 3 days. Growth curves for this series are given in Fig. 1.

That the effect of MSG in increasing growth of mice on this amino acid ration is due to increased food consumption is evident from the data in Table II. Figures are presented for only the first 10 days since it has been found that after this time the differences in

food consumption between the various groups are relatively constant. The utility of MSG in increasing food consumption was most evident during the first 7 days. After this time the food consumption of the unsupplemented group increased to a level comparable with the MSG groups. Although completely equivalent food consumption on the casein and amino acid rations has not been obtained by the use of MSG, it appears likely that the maximum effect obtainable with this substance and this particular amino acid ration has been reached since 2% MSG did not give

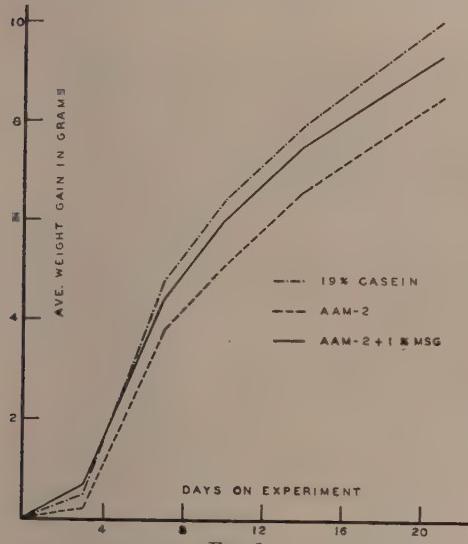


FIG. 1

Growth of mice fed rations containing casein, amino acids or amino acids plus MSG.

TABLE II. Food Consumption Data.

Day	Avg food consumption per mouse per day, g			
	19% casein	AAM-2	AAM-2 + 1% MSG	
1	2.9	2.1	2.0	2.3
2	4.1	2.9	3.5	2.9
3	4.1	2.9	3.5	2.9
Days 1-3 avg	3.7	2.6	3.0	2.7
4	4.2	3.6	4.1	3.9
5	4.6	3.3	3.1	3.2
6	4.8	3.7	4.6	4.7
7	4.8	4.3	4.6	5.0
Days 4-7 avg	4.6	3.7	4.1	4.2
8	4.5	4.3	3.7	4.7
9	3.9	3.6	3.5	3.3
10	5.4	4.4	5.0	4.6
Days 8-10 avg	4.6	4.1	4.1	4.2
Avg/day 10 days	4.3	3.5	3.8	3.8

significantly better results than 1%.

**Summary.** A synthetic ration in which all of the nitrogen is furnished as free amino acids is not as palatable to the mouse as one containing casein. The inferior palatability results in less food consumption during the first 3 to 7 days and consequently poorer growth. When the initial lag period is diminished or eliminated by the addition of 1% of monosodium glutamate to the ration, the final weights of the mice are more consistent and approach more closely those of the animals fed a ration containing casein.

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## Purification of the Egg-White Inhibitor of Influenza Virus Hemagglutination by Filtration.\* (18115)

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Egg-white (EW) is one of the richest and

most readily available sources of a material capable of inhibiting hemagglutination by heated influenza viruses(1). This material has considerable interest as a substrate for the enzyme-like action of influenza viruses

\* This work was supported by a research grant from the National Cancer Institute, U. S. Public Health Service, and through the Commission on Influenza, Armed Forces Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, D. C.

1. Lanni, F., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 312.

(2-5). One of the principal problems is the preparation of highly purified inhibitor in large quantity. The method usually employed in this laboratory(6) consists in the precipitation of the inhibitor from EW at pH 5.7 and extraction of the precipitate, after washing, with phosphate buffer of pH 7.2. The preparations obtained in this way are usually 40-60 times as active as EW on a nitrogen basis.

In this paper there is presented a new and very simple method, which yields inhibitor solutions about 80 times as active as EW. The method consists in filtration of diluted EW through rapid-flow filter papers and extraction of the inhibitor from the paper. Furthermore, the method is capable also of increasing the purity of preparations obtained by acid-precipitation.

*Materials and methods.* Thick EW for the filtration experiments was separated from fresh eggs with the aid of a screen, strained through cotton gauze, and poured into 9 volumes of 0.06 M phosphate buffer of pH 7.2; by gentle stirring for about 2 hours, a fairly homogenous solution was obtained. Semipurified EW inhibitor was prepared from undiluted thick EW by acid-precipitation as described before(6); some of the extracts were concentrated 4-5 times by prevaporation and were used as such in the filtration experiments. All materials were preserved with Merthiolate (Lilly), which was present in a concentration of 1:5,000 throughout the purification procedures. Filtrations were carried out in a wet chamber at 4°C. Inhibitory activity(6) and nitrogen(7) were determined<sup>†</sup> by the usual methods of this laboratory. The reported inhibition titers

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TABLE I. Properties of Fractions Obtained by Filtration of Thick EW.

Fraction	Inhibition titer	N, $\mu\text{g}/\text{ml}$	PF	Activity, %	Recovered from starting material
Thick E W 1:10	7500	1750	1.1		
Filtrate	190	1700	0.03	2.5	97.0
Extract I	280	44	1.6	3.7	2.5
" II	6000	18	83	80.0	1.0
Total				86.2	100.5

have been standardized by reference to a preserved solution of EW (EW Standard I) (8). The purification factor (PF) of a preparation is the ratio of the specific activity (titer/nitrogen) of the preparation to that of the standard EW.

*Experimental. Thick EW.* The results obtained by filtration of thick EW are illustrated in the findings of the following experiment: 500 ml of diluted (1:10) thick EW was poured onto a 32-cm folded filter paper (Reeve Angel No. 802).<sup>‡</sup> A volume of 473 ml of filtrate was collected in 32 hours. The unfiltered portion of the solution was discarded, and the filter paper was unfolded and washed briefly with 470 ml of phosphate buffer (0.06 M, pH 7.2) by gentle swirling (Extract I). The paper was then put, together with 470 ml of fresh buffer, in a Waring Blender and shredded by gentle blending for about 5 minutes. The mash was slowly stirred at room temperature for 24 hours and centrifuged; a viscous, opalescent supernatant was obtained (Extract II). Table I shows the relation between inhibitory activity and nitrogen at different steps.

*Semipurified Inhibitor.* The experiments were carried out as with thick EW. Illustrative results, obtained with 2 preparations

† The authors are grateful to Mrs. Edith S. Dillon for the nitrogen analyses.

‡ Other rapid filter papers (e.g., Whatman No. 1 and No. 4) could be substituted in the purification procedure.

TABLE II. Properties of Fractions Obtained by Filtration of Acid-Purified Inhibitor.

	Preparation					
	Titer	C20-PEIII N, µg/ml	PF	Titer	C21-PEIII-CII N, µg/ml	PF
Starting material	12000	168	18	33000	145	57
Filtrate	260	109	0.6	190	54	0.9
Extract I	4000	31	32	8900	31	72
" II	5200	18	72	5400	18	75
Filter paper used	Reeve Angel #802					
	Whatman #1					

(C20-PEIII and C21-PEIII-CII) of acid-purified inhibitor, are shown in Table II. It is seen that the levels of PF of the 2 preparations, which differed widely before filtration, were increased to approximately the same value and one similar to that obtained by direct purification of the inhibitor by filtration of thick EW (Table I).

*Discussion.* Several alternative methods have previously been employed for purification of the EW inhibitor. The method(6) based on precipitation of the inhibitor at pH 5.7 and extraction at pH 7.2 has proved very useful for the processing of relatively small volumes (100-200) ml of EW. By this method, useful preparations, with PF in the range 40-60, can be obtained in 4-5 hours, since no prolonged period is needed for precipitation or extraction of the precipitate, and dialysis is not required. With the processing of larger volumes of EW, the method becomes cumbersome. Although acid-purified preparations have been shown to contain 3 related components, the properties of which are discussed elsewhere(9,10), electrophoretic analysis failed to reveal any lysozyme. However, a quantity of lysozyme amounting to less than 10% of the total protein would have been difficult to discern electrophoretically; no biological tests for lysozyme in these preparations have been carried out.

Some acid-purified inhibitor preparations precipitate in the Tiselius cell during electrophoresis. The precipitate contains about 60% of the original activity with considerable increased PF(11). The use of this method

in routine purification procedures is limited by the capacity of the Tiselius cell.

A second method, employed by Gottschalk and Lind(12) and based on the method described by Young(13) for the purification of ovomucin, consists in the precipitation of the inhibitor by dilution of EW with distilled water at 0°C. The precipitate is washed in the cold, dispersed in 5% NaCl solution, and dialyzed for 48 hours at 4°C to bring the salt concentration to 1%. The method is thus rather gentle, but a disadvantage consists in that it requires laborious handling of the material in the centrifuge. The final product contains up to about 15% of lysozyme, part of which may be removed by crystallization. No values for the yield or PF have been reported; however, in a limited experience of the authors (unpublished) the method yielded preparations with PF 30-40, with the lysozyme not specifically removed.<sup>§</sup> The preparations are reported to contain at least one inert component in addition to lysozyme(4).

A third method, based on centrifugation of the inhibitor from diluted EW at high speed(6), has yielded products with PF in the range 40-50. The method has not been extensively explored, because of the need for specialized apparatus and the availability of other methods.

Finally, it has recently proved possible to obtain considerable further purification of

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13. Young, E. G., *J. Biol. Chem.*, 1937, v120, 1.

<sup>§</sup> In a personal communication, Dr. Gottschalk has informed the authors that the PF of his preparations is approximately 40-50.

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10. Sharp, D. G., Lanni, F., Lanni, Y. T., Csáky, T. Z., and Beard, J. W., submitted for publication.

semipurified inhibitor by isolation of the fast-moving, active component in the electrophoresis apparatus(10). A preparation with PF 190 was obtained from a starting material with PF 44. The yield of such highly purified inhibitor by this method is still smaller than that obtained through the use of electrophoretic precipitation (cited above).

In relation to these methods, the method based on filtration possesses the distinct advantages that (a) the procedure is extremely simple, (b) the conditions are mild, (c) no special apparatus is required, (d) the capacity is large, and (e) the products are superior in PF to all except those obtained by electrophoretic isolation; disadvantages are (a) the products are dilute and, for some uses, must be concentrated by pervaporation, with subsequent dialysis to reduce the salt concentration, and (b) the preparation of a batch of in-

hibitor requires at least 3 days, 2 for filtration and 1 for extraction. Thus far, attempts to reduce the time of filtration or extraction have proved unsuccessful.

The conditions affecting the filtration properties of the inhibitor have been studied extensively in experiments which will be reported separately.

**Summary.** A simple method is described for the purification of the egg-white inhibitor of influenza virus hemagglutination by filtration of diluted egg-white through rapid-flow filter papers and extraction of the unfilterable residue. The inhibitor has been purified about 80-fold in this way. The method has proved useful also for the further purification of acid-purified inhibitor. The advantages and disadvantages of the new method are discussed in relation to those of other methods.

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## Failure to Find C-Reactive Protein in Viral Hepatitis.\* (18116)

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(Introduced by Abraham Cantarow)

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The presence of C-reactive protein in the blood of patients with a wide variety of diseases has been described(1-4). Although the exact mechanism which potentiates the appearance of this substance is not clearly defined, it has been suggested that several different and apparently non-specific stimuli producing damage to tissues may be operative. Thus, C-reactive protein has been

found in the blood of patients with infarction of the myocardium, acute and chronic infectious diseases, and following the intramuscular injection of such materials as vaccines and colloidal sulphur(5). Although frequent observations have been made of the appearance of C-reactive protein in the blood of patients with bacterial infections, only a limited amount of data is available concerning the presence of this substance in the blood of patients with viral diseases. It has been described(4) as occurring in occasional patients with influenza, viral hepatitis, infectious mononucleosis, poliomyelitis, mumps, and following vaccination for small-pox, but Löfström(3) reported that C-reactive protein was uncommonly present in the blood of patients

\* This investigation was conducted with the aid of the Commission on Virus and Rickettsial Diseases, Armed Forces Epidemiological Board, Office of The Surgeon General, U. S. Army, Washington, D. C.

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2. Ash, R., *J. Infect. Dis.*, 1933, v53, 89.

3. Löfström, G., *Acta med. Scandinav.*, *Supplement 141*, 1943.

4. Hedlund, P., *Acta med. Scandinav.*, *Supplement 196*, 579, 1947.

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with viral infections unless secondarily invaded by bacteria.

During recent efforts to devise a diagnostic test for viral hepatitis, an apparently non-specific serologic reaction was encountered(6). A large percentage of one group of patients with the disease had in their serums during the acute phase a substance which reacted with collodion particles in such a way that they were agglutinated by convalescent hepatitis gamma globulin. This substance was associated with the globulin fraction of the serum and, although the exact mechanism of the reaction was not defined, it was thought to be unrelated to hepatitis virus. In the attempt to elucidate the nature of this reaction, a large number of these serums were tested for the presence of C-reactive protein, and it is the purpose of this paper to report the results of these studies.

*Methods and materials. Serums.* Serums which were utilized in the tests were obtained from 90 patients with viral hepatitis in an American Army hospital in Germany. It is quite probable that many of these cases were examples of homologous serum hepatitis but, in view of the fact that there are no means of differentiating infectious hepatitis from serum hepatitis clinically, the cases have all been classified as viral hepatitis. Some of the serums were frozen at once and stored at a temperature of  $-4^{\circ}\text{C}$  for periods ranging from one to 4 weeks. They were then shipped to the United States in containers with dry ice, and all serums were stored at dry ice box temperature for periods of one to 4 months before use. Other serums were tested within 2 hours after being obtained from the patients. For purposes of control, serums were obtained from patients with other acute infectious diseases including lobar pneumonia.

*Technic of test.* The test was performed according to the method of Abernethy and Francis(7), and dilutions of C-polysaccharide<sup>†</sup> ranging from 1:5000 to 1:640,000 were used.

*Results and comment.* The results of the

TABLE I. Results of Tests for C-Reactive Protein in Serums of 90 Patients with Viral Hepatitis.

Days of disease	No. of serums	
	Tested	Positive
5-7	7	0
8-10	33	0
11-14	32	0
15-21	9	0
22-28	4	0
29-63	5	0
Total	90	0

determinations in the 90 patients are tabulated according to phase of disease in Table I. The uniformly negative reactions are slightly different from the results of Hedlund (4) who found 3 positive serums among 26 patients with viral hepatitis; 2 of these were obtained in the pre-icteric phase of disease. Almost half of the patients in the group described here were tested in the first 10 days of disease, and the large majority were examined within the first 2 weeks. Many of these serums contained greater than normal amounts of bilirubin, although a few of the earliest obtained had normal amounts of bilirubin.

It is not known why C-reactive protein should be found so uncommonly in the blood of patients with viral hepatitis in contrast to its frequent appearance in the blood of patients with bacterial disease such as lobar pneumonia. It is possible that an insufficient number of patients were examined in the very early phase of disease. However, in view of the pathogenesis of viral hepatitis, it is of interest that such uniformly negative results were obtained if the mechanism of production of C-reactive protein depends, as has been hypothesized, entirely on the nonspecific destruction of tissue(8). Although hepatic necrosis occurs before the appearance of icterus in patients with viral hepatitis, it often continues into the second week of disease or even longer in certain patients. Occasional patients studied later in disease had active hepatitis. The fact that these tests were also negative is of particular interest in

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<sup>†</sup> This was kindly supplied by Dr. Colin M. MacLeod, New York University College of Medicine.  
8. Löfström, G., *Brit. J. Exp. Path.*, 1944, v25, 21.

relation to the observations of Anderson and McCarty(9) on the value of the presence of C-reactive protein as an indicator of activity of disease in rheumatic fever.

The possibility must also be considered that the method of testing for C-reactive protein was not sufficiently sensitive to determine small amounts which might have been more readily detected by the use of C-protein antibody(10,11). However, the fact that the results were uniformly negative in contrast to the frequency of positive tests by the same method in various bacterial diseases raises the question as to whether this may be an expression of a fundamental difference in the pathogenesis of certain viral infections. It is noteworthy that most of the patients described here had an insidious onset of hepatitis with little or no fever. Although there is apparently no constant relationship between fever and the presence of C-protein in the blood, the association of fever, increase in alpha globulins and the presence of C-protein has been described in various other conditions. The alterations of serum proteins are of interest in this regard, and electrophoretic separation of serums containing C-reactive protein has revealed that it is associated with the alpha globulin fraction(12). It is known that this fraction is increased early in lobar

pneumonia(13), while in viral hepatitis the beta and gamma globulins are more frequently increased, although the alpha fraction may also be augmented in occasional patients(14-16).

**Summary.** The serums of 90 patients in various phases of viral hepatitis were tested for the presence of C-reactive protein, and the results were uniformly negative. The significance of this is undetermined but the question is raised as to whether it may be an expression of a fundamental difference in the pathogenesis of certain viral infections.

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## Production of Specific Antisera Against Sickle Cell Anemia Erythrocytes; Antibody in Sicklemia Sera.\* (18117)

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**A. Production of specific rabbit antisera against sickle cell anemia erythrocytes.** During the course of experiments on the carbonic anhydrase content of erythrocytes from sickle cell anemia patients(1) it was noted that

when such erythrocytes were diluted 1:100 or 1:200 with water, marked cloudiness appeared, in sharp contrast to the clear appearance of the same dilutions of normal erythrocytes. Similar but generally less marked clouding was often noted with erythrocytes from individuals with sickle cell trait. The well known resistance of sickling erythrocytes to hemolysis in hypotonic solutions was con-

\* Aided by a grant from the American Cancer Society, CP-12B, administered by C. M. Pomerat.

1. Schneider, R. G., Levin, W. C. and Haggard, M. E., *J. Lab. and Clin. Med.*, 1949, v34, 1249.

sidered the possible explanation of the cloudiness, but microscopic examination of the centrifuged sediment revealed only amorphous material and no intact cells. Furthermore, the cloudiness did not disappear after prolonged standing, as would be expected with delayed hemolysis. When the sediment was centrifuged, washed repeatedly with water and analyzed, the nitrogen content was much higher than would be expected from the protein content of the stroma, after correction for the adsorbed hemoglobin(2). It was believed, therefore, that a protein was probably responsible for the clouding.

An attempt was made to determine whether sickle cell anemia erythrocytes are distinguishable immunologically from normal erythrocytes.

*Methods.* Erythrocytes from oxalated blood were washed three or four times in isotonic saline solution. After the final washing the cells were diluted with isotonic saline containing 0.12% of dextrose-sodium-citrate-citric-acid solution, so that 1.0 ml of the final suspension contained 0.1 ml of packed cells. When suspensions were used for more than three injections, a drop of 1:1000 aqueous merthiolate was added as preservative. Six rabbits were used for injection: 4 received erythrocytes from sickle cell anemia patient O.W. (OMNRh+), and 2 rabbits erythrocytes from sickle cell anemia patient J.A.G. (BNRh+). The animals were injected intravenously with 0.1 ml of cell suspension for 3 consecutive days. After a 4 day rest period they were reinjected for 3 consecutive days, and again allowed to rest for 4 days. Seven to 10 days after the fourth course of injections, the animals were bled. Thereafter they received a booster injection at approximately monthly intervals and sera were collected 7 days after each injection. The sera were inactivated at 56°C for 30 minutes, diluted 1:20, and absorbed. Absorptions were performed by adding to the diluted serum half the volume of washed packed erythrocytes of the appropriate blood group and type. The mixture was allowed to

remain at room temperature 30 minutes, the cells centrifuged off, and the process repeated. Generally, 2 absorptions were found to be sufficient, although occasionally a third was necessary. The sera were stored in small vaccine bottles in a frozen state. In most experiments no preservative was added. This necessitated careful attention to the possibility of contamination and consequent development of non-specific agglutinins. Towards the end of this study sodium azide was added to the thawed sera to make a concentration of 0.1%, as has been suggested for the preservation of Rh antisera(3). Agglutination tests were performed by mixing 0.05 ml of serum with 0.05 ml of a 2% suspension of washed red cells. The tubes were allowed to stand at room temperature for one to 3 hours, and readings made under a dissecting microscope. The tubes were placed in the refrigerator overnight, after which final readings were made. Clumps visible to the naked eye were graded 2 to 3+. Clumps visible only under the dissecting microscope were graded 1+. In doubtful cases the tubes were centrifuged at about 750 r.p.m. for 1½ minutes before the final reading. An occasional cold agglutinin appeared after refrigeration, so that all tubes were allowed to come to room temperature before reading. Results were found more dependable when the erythrocytes were obtained from freshly drawn blood.

All individuals with the diagnosis of sickle cell anemia had been thoroughly studied in this hospital. Individuals with sickle cell trait were obtained by means of sickling surveys made periodically on all Negro patients. An effort was made to obtain test cells before patients had undergone transfusion, but this was not always possible, particularly among the individuals with sickle cell trait, some of whom had been admitted for surgery. Control erythrocytes were usually obtained from the blood bank. Most of the control cells were from white donors, but thirteen were from Negroes (not tested for the presence of sickling).

2. Schneider, R. G., and Levin, W. C., unpublished data.

3. Batson, H. C., Jayne, M., and Brown, M., *J. Lab. and Clin. Med.*, 1950, v35, 297.

## IMMUNOLOGICAL STUDIES OF SICKLEMIA

*Results.* Of the 6 animals injected, 5 produced usable sera. Most of the tests were performed with sera from rabbits 319 and 320, injected with cells from patient J.A.G. (BNRh+) and from rabbit 323, injected with cells from patient O.W. (OMNRh+).

Table I shows the results of agglutination reactions with these sera against cells from 124 normal individuals, 19 individuals with sickle cell anemia, and 21 with sickle cell trait. Only those erythrocytes from patients with sickle cell anemia were agglutinated by the sera. None of the erythrocyte suspensions from individuals with sickle cell trait reacted with the sera. Two most likely explanations of this finding were considered: (1) That this was due to insufficient antibody in the sera to produce visible agglutination with the trait cell; (2) that the antibody was specific for sickle cell anemia erythrocytes. In order to test these explanations, absorption experiments were performed with cells from individuals with sickle cell trait and from those with sickle cell anemia. To the sera, absorbed as described previously, was added one-half the volume of washed, packed erythrocytes from individuals with the trait or the anemia. After standing 30

TABLE I. Summary of Agglutination Reactions of Anti-Sickle Cell Anemia Rabbit Sera with Erythrocytes of Normal Individuals and Those with Sickle Cell Anemia and Sickle Cell Trait.

Scrum from rabbit No.	No. of indi- viduals tested	Diagnosis	% positive
323	82	Normal	0
	17	Sickle cell trait*	0
	16	," " anemia	100
320	59	Normal	0
	13	Sickle cell trait	0
	19	," " anemia	100
319	100	Normal	0
	15	Sickle cell trait	0
	19	," " anemia	100
All three sera	124	Normal	0
	21	Sickle cell trait	0
	19	," " anemia	100

\* Two of these patients were originally diagnosed as sickle cell anemia but re-evaluation of the clinical data makes the diagnosis of sickle cell trait more likely. A more detailed report of these patients will be made at a later date.

TABLE II. Absorption of Anti-Sickle Cell Anemia Rabbit Sera with Erythrocytes from Individuals with Sickle Cell Trait.

Date of absorption	Serum No.	Agglutination reaction with erythrocytes of patients with sickle cell anemia after ab- sorption of sera with eryth- rocytes of patients with sickle cell trait
3/23	320	++
5/8	323	+
5/24	323	+
		-
5/12	323	++
		+
5/29	323	++
		+

TABLE III. Absorption of Anti-Sickle Cell Anemia Rabbit Sera with Erythrocytes from Individuals with Sickle Cell Anemia.

Date of absorption	Serum No.	Agglutination reaction with erythrocytes of patients with sickle cell anemia after ab- sorption of sera with eryth- rocytes of patients with sickle cell anemia
3/23	320	-
		-
3/28	319	-
5/24	323	-

minutes at room temperature, the tubes were centrifuged, the serum drawn off, and the process repeated.

Table II demonstrates that absorption of sera with cells from sickle cell trait does not remove the agglutinins for sickle cell anemia erythrocytes. Of the eleven sickle cell anemia cell suspensions tested against various sera absorbed with sickle cell trait erythrocytes only one reaction (C.W.) was negative. This might well be explained by non-specific absorption.

Table III demonstrates that absorption of the sera with erythrocytes from individuals with sickle cell anemia completely removes all agglutinins in nine tests with erythrocyte suspensions of sickle cell anemia patients. It appears, therefore, that the anti-sickle cell

TABLE IV. Titrations of Anti-Sickle Cell Anemia Rabbit Sera 319, 320, 323 Against Erythrocytes from Individuals with Sickle Cell Anemia.

Dilutions X 20	Patient P.R.	Patient S.S.	Patient O.W.	Patient D.G.J.	Patient C.W.
<b>Serum 319</b>					
und.	+	++	++	+	++
2	++	++	++	++	++
4	++	++	++	++	++
8	++	++	++	++	++
16	++	++	++	++	++
32	++	++	++	++	++
64	++	++	++	++	++
128	++	++	++	++	++
<b>Serum 320</b>					
und.	++	++	++	++	++
2	++	++	++	++	++
4	++	++	++	++	++
8	++	++	++	++	++
16	++	++	++	++	++
32	++	++	++	++	++
64	++	++	++	++	++
128	++	++	++	++	++
<b>Serum 323</b>					
und.	+	++	++	++	++
2	++	++	++	++	++
4	++	++	++	++	++
8	++	++	++	++	++
16	++	++	++	++	++
32	++	++	++	++	++
64	++	++	++	++	++
128	++	++	++	++	++

anemia serum contains an antibody specific for sickle cell anemia erythrocytes.

Titrations of rabbit sera 319, 320, and 323 against erythrocytes from several sickle cell anemia patients are given in Table IV. The most frequently observed titer is 1-16 (times 20). The lowest is serum 319 against patient, S.S., positive only when undiluted (actually 1:20).

B. *Demonstration of agglutinins in sickle cell anemia and sickle cell trait.* An attempt was made to determine whether an agglutinin exists in the sera of individuals with sickle cell anemia and sickle cell trait.

*Methods.* Erythrocytes from oxalated blood were washed 3 to 4 times with isotonic saline. One-tenth milliliter of a 2% suspension of cells, either in saline or in 20% bovine albumin (Armour) was mixed with 0.1 ml of the patient's serum. Tubes were placed in the water bath at 38° for one hour, then centrifuged at 750 r.p.m. for 1½ minutes. Readings were made as described previously.

Tests were also made to determine the presence of antibody (human globulin) adsorbed to the erythrocytes, according to the method of Coombs, Mourant, and Race(4). The "direct" Coombs test was used, and final readings were made after refrigeration for 18 hours.

*Results* of agglutinin tests on the sera of 13 patients with sickle cell anemia are shown in Table V. Saline or albumin agglutinins are present in the sera of all but one of these 13 patients. All gave evidence of the presence of antibody adsorbed on the erythrocytes, by a positive reaction with the antiglobulin (Coombs) test.<sup>†</sup> Of the 11 individuals with sickle cell trait, 3 show saline agglutinins, four show agglutinins in albumin, while 2 have doubtful albumin agglutinins. All of our sickle cell anemia patients had received numerous transfusions. Some of the patients with sickle cell trait had been transfused for unrelated diseases. The possibility that agglutinin formation was related to repeated transfusions was considered. Accordingly, patients with no evidence of sickling, but who had had numerous transfusions were also examined for the presence of agglutinins. To date, six such patients have been tested for the presence of agglutinin. All have been negative.

Investigations are under way to determine whether the specific antigen of the sickle cell anemia erythrocyte is contained in the stroma or in the hemoglobin. The fact that it appears to be specific for sickle cell anemia would indicate that it is not related to the abnormal hemoglobin found by Pauling(5) to be present in erythrocytes with the sickling capacity. Pauling describes a quantitative difference between erythrocytes from individuals with sickle cell anemia and those with sickle cell trait, in that the former contain 100% of the abnormal hemoglobin, while the latter contain a mixture of about 50% of

4. Coombs, R. R. A., Mourant, A. E., and Race, R. R., *Brit. J. Exper. Path.*, 1945, v26, 255.

† We are grateful to Dr. J. M. Hill and Dr. Sol Haberman, Baylor Hospital, Dallas, Texas, for supplying some of the antiglobulin serum.

5. Pauling L., Itano, H. A., Singer, S. J. and Wells, I. C., *Science*, 1949, v110, 543.

TABLE V. Agglutinins in Sera of Individuals with Sickle Cell Anemia and Sickle Cell Trait.

Erythrocytes of:	Diagnosis*	Saline suspensions	Albumin suspensions	Antiglobulin (Coombs) test
W.B.	SCA	—	—	+
P.R.	"	+	—	++
N.N.	"	++	+	++
J.W.	"	++	—	++
A.B.	"	++	+	++
D.W.	"	—	++	—
S.S.	"	++	++++	++
D.G.J.	"	—	+	—
M.S.	"	—	+	++
W.K.	"	—	+	—
W.E.	"	—	++	—
C.W.	"	—	++	—
O.W.	"	—	+	++
I.C.G.	T	—	++	—
M.B.	T	—	++	—
W.M.	T	—	—	—
G.E.	T	—	—	—
O.W.	T	—	—	—
A.S.	T	—	—	—
C.M.	T	—	—	—
F.T.	T	—	—	—
D.K.	T	—	—	—
C.J.	T	—	—	—
S.J.	T	—	+	—

\* SCA indicates sickle cell anemia.

T " " " trait.

the abnormal hemoglobin and about 50% normal hemoglobin. Our results would indicate that there is a *qualitative* difference in the antigenic structure of erythrocytes from individuals with sickle cell trait and those with sickle cell anemia.

The demonstration of agglutinins in the sera of all the thirteen sickle cell anemia patients tested suggests that the hemolytic pro-

cess in sickle cell anemia may be related to the presence of agglutinin in the patients' sera. Further experiments are in progress to determine the nature of this agglutinin and the significance of its presence in about one-third of our cases of sickle cell trait. Since our rabbit sera have given absolute differentiation between the sickling trait and the anemia, we believe that anti-sickle cell anemia rabbit sera may offer a diagnostic aid in the differentiation of sickle cell anemia from sickle cell trait in those cases in which the latter is complicated by other forms of anemia, and presents a difficult diagnostic problem.

*Summary.* 1. Erythrocytes from 2 individuals with sickle cell anemia, when injected into rabbits, produced antisera which agglutinated erythrocytes of 19 individuals with sickle cell anemia, but not those of 21 individuals with sickle cell trait nor those of 124 normal individuals.

2. Such sera may be useful in the differential diagnosis between sickle cell anemia and sickle cell trait.

3. The presence of agglutinin antibody has been demonstrated in the sera of all of 13 patients with sickle cell anemia. This finding may explain the hemolysis which is characteristic of this disease.

4. Of 11 patients with sickle cell trait, definite agglutinin antibody was demonstrated in the sera of 4, and doubtful agglutinin antibody in 2.

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### Effects of HCl on Alkaline Phosphatase in Kidney and Intestine: Histochemical and Quantitative Study.\* (18118)

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Numerous histochemical and biochemical studies have demonstrated the widespread dis-

tribution and specific location of alkaline phosphatase in various tissues. However, the

\* An abstract covering part of this data appears in *Anat. Rec.*, 1949, v103, 29.

1. Bodansky, O., *J. Biol. Chem.*, 1937, v118, 341.  
2. Cloetens, R., *Enzymologia*, 1939, v6, 46.

identity or diversity of the enzymes at these sites remains an unsettled question(1-8). It has been shown(10) that in the mouse, renal phosphatase may be distinguished from intestinal phosphatase by the greater sensitivity of the latter to inhibition by cyanide. Present observations on the effects of HCl provide a further basis for distinguishing between the alkaline phosphatases of these two organs.

*Materials and methods.* A. *Histochemical.* Specimens of kidney and intestine, obtained from guinea pigs and albino mice killed by decapitation and from a dog under nembutal anesthesia, were fixed in chilled acetone(9). Sites of alkaline phosphatase activity were demonstrated by the method of Gomori(11).

To study the effect of increasing concentrations of HCl on histochemically demonstrable phosphatase, slides bearing sections of both kidney and intestine were placed for  $\frac{1}{2}$  hour at 37°C in solutions of HCl ranging from pH 5.0 to 1.8. After rinsing in distilled water the entire group of slides, each of which had been subjected to a different pH, was then carried simultaneously through the procedure for demonstrating the sites of phosphatase activity.

B. *Quantitative.* Kidneys, and segments of intestine rinsed with saline, were homogenized in chloroform water, allowed to autolyze at room temperature for 12 to 24 hours, then reground and partially clarified by brief centrifugation. The concentration of the supernatant thus obtained was so adjusted that 0.5 cc of a 1:10 dilution gave a suitable colorimeter reading when incubated for one

3. Roche, J., de Laromiguiére, S., and Laurens, A., *Compt. rend. Soc. de Biol.*, 1943, v87, 243.

4. Dempsey, E. W., and Deane, H. W., *J. Cell. and Comp. Physiol.*, 1946, v27, 159.

5. Gomori, G., *Proc. Soc. EXP. BIOL. AND MED.*, 1949, v70, 7.

6. Bodansky, O., *J. Biol. Chem.*, 1949, v179, 81.

7. Gomori, G., *Ann. N. Y. Acad. Sci.*, 1950, v50, 968.

8. Newman, W., Feigin, I., Wolf, A., and Kabat, E. A., *Am. J. Path.*, 1950, v24, 257.

9. Emmel, V. M., *Anat. Rec.*, 1946, v95, 159.

10. Emmel, V. M., *Anat. Rec.*, 1946, v96, 423.

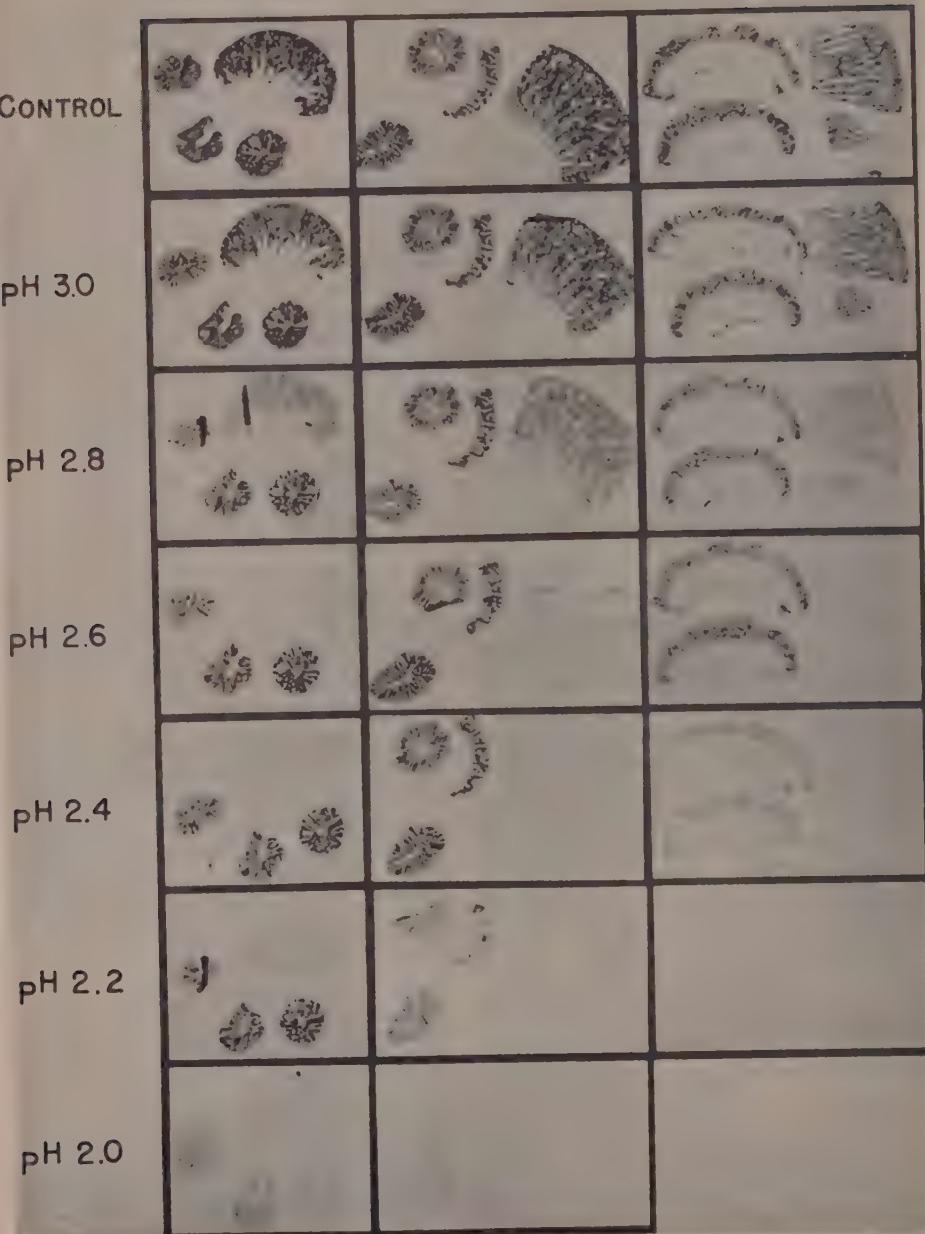
11. Gomori, G., *Amer. J. Clin. Path.*, 1946, v16, 347.

hour at 37°C in phenolphthalein phosphate substrate(12). Test tubes containing 5.0 cc of HCl solutions ranging from pH 4.5 to 1.8 were warmed to 37°C. To each was added 0.5 cc of homogenate. After  $\frac{1}{2}$  hour duplicate 0.5 cc aliquots of each HCl-homogenate mixture were removed for phosphatase determination(12). To ascertain the pH at which the homogenates had been treated, the pH of the remainder of each HCl-homogenate mixture was then determined with a Beckman pH meter. The pH of a 5.0 cc portion of the substrate was lowered only 0.2 pH unit (9.6 to 9.4) by the addition of 0.5 cc of the most acid of these mixtures. The amount of phosphatase activity surviving treatment with the various concentrations of HCl was calculated in arbitrary units.

*Results.* Typical results obtained when tissues from dog, mice, and guinea pig were subjected to acid treatment before staining for phosphatase are shown in Fig. 1. From these histochemical observations it is apparent that in all three species the alkaline phosphatase of the kidney is inactivated by a lower concentration of HCl than is the corresponding enzyme of the intestine.

In agreement with the histochemical observations, the quantitative data (Fig. 2) demonstrate that in crude homogenates renal alkaline phosphatase of the mouse is inactivated at a distinctly higher pH than is intestinal phosphatase. The slight rise in activity of intestinal phosphatase following treatment at pH 3.8 to 2.8 was a constant finding. The first portion of the upper curve in Fig. 2 approximates a summation of the individual curves for kidney and intestine, but beyond pH 3.0 the curve is appreciably elevated above that for the intestine alone. In a preliminary investigation of this phenomenon(13) it was found that acid-inactivated renal homogenate (pH 2.5 for  $\frac{1}{2}$  hour, then neutralized) added to intestinal homogenate increased the resistance of the latter to acid inactivation by approximately the amount apparent in Fig. 2. On the other hand, acid-inactivated intestinal homogenate (pH

12. Huggins, C., and Talalay, P., *J. Biol. Chem.*, 1945, v159, 399.

**MOUSE      G. PIG      DOG**
**CONTROL****pH 3.0****pH 2.8****pH 2.6****pH 2.4****pH 2.2****pH 2.0****FIG. 1.**

Acid inactivation of alkaline phosphatase in kidney and intestine of mouse, guinea pig, and dog. Figures on left indicate pH of HCl to which specimens were subjected at 37°C for  $\frac{1}{2}$  hour prior to treatment with Gomori technic. Degree of blackening is related to amount of phosphatase activity. Not counterstained. Dark streaks in guinea pig kidney at pH 2.6 are due to folds in the section.

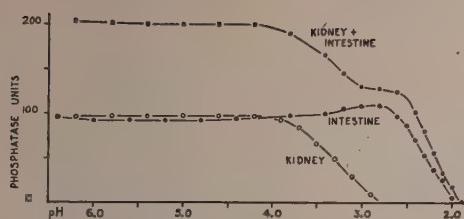


FIG. 2.

Acid inactivation of alkaline phosphatase in homogenates of mouse kidney and intestine and in a homogenate containing equivalent amounts of each. Abscissa indicates pH at which homogenates were treated at 37°C for  $\frac{1}{2}$  hour prior to determination of phosphatase activity. Enzyme units are arbitrary. Each curve represents an average of 3 experiments.

1.8 for  $\frac{1}{2}$  hour, then neutralized) slightly decreased the resistance of renal homogenate to acid inactivation.

**Discussion.** A problem which arises in the interpretation of histochemical studies of enzyme inhibitors(5,7) is that local differences in concentration of a single enzyme may be misinterpreted as indicating differences in sensitivity or resistance to a given agent. For example, in the action of cyanide on mouse kidney and intestine(10), it might be supposed(5) that the apparently greater resistance of renal phosphatase is due merely to a higher enzyme concentration in this organ. If such a quantitative difference were the only factor, then with any agent capable of suppressing phosphatase activity the kidney should always be found more resistant than the intestine. The fact that renal phosphatase is actually less resistant to acid inactivation renders such a simple interpretation untenable; since on the basis of the cyanide effects it would be necessary to assume a higher enzyme concentration in the kidney, and on the basis of the HCl effects it would be necessary to assume a higher concentration in the intestine. It thus becomes justifiable to conclude that in addition to whatever quantitative differences there may be in the phosphatase content of these two organs, there must also be qualitative differences.

It should be pointed out that the degree

of difference between kidney and intestine may vary with species. The difference in HCl sensitivity between renal and intestinal phosphatase is greater in the mouse than in the dog (Fig. 1). Species variation also occurs with respect to cyanide sensitivity(10): in mouse the intestine is more sensitive than the kidney, while in dog(13) both organs are about equally sensitive. Gomori(5) has implied that in some species (not identified) the kidney may be more sensitive to cyanide than is the intestine.

The chemical basis for the differences between the alkaline phosphatases of these two organs is not understood. In crude homogenates the presence of substances acting as inhibitors, activators, or protective agents might contribute to the observed differences in HCl sensitivity. However, the curve obtained with the mixed homogenates indicates that whatever effects such substances may have, the renal and intestinal phosphatases retain their individuality in the presence of one another. The fact that the HCl sensitivity of the alkaline phosphatase in fresh tissue homogenates closely approximates that demonstrable histochemically in specimens which have been fixed, washed and subjected to fat solvents would seem to minimize the importance of dializable substances in the homogenates and suggests that the differences may lie in the enzymes themselves, or be due to something which remains firmly associated with the enzyme even during the rigors of histological preparation.

**Summary.** The inactivation of renal and intestinal alkaline phosphatases by HCl was studied by histochemical and by quantitative methods. In the mouse, guinea pig and dog the renal enzyme is inactivated at a distinctly higher pH than is the intestinal enzyme. These observations support the view that renal and intestinal alkaline phosphatases are not identical.

13. Emmel, V. M., unpublished data.

## Lipotropic Agents in Liver Damage Produced by Selenium or Carbon Tetrachloride (18119)

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The severity of liver damage produced by the prolonged ingestion of small amounts of toxic materials is known to be influenced by the nature of the diet. The relationship is complicated by a variety of factors, some of the more important being species, sex, rate of growth, the physiological state of the animal at the time of study, caloric intake, and other complex conditions such as multiple rather than single nutritional deficiencies. Another important consideration is the possibility of chemical or pharmacological interaction between a dietary factor and the poisonous substance.

The purpose of the present study was to examine the effects of giving supplements of choline, of methionine or of cystine to rats which were fed an "adequate" diet and in which hepatic damage was produced by the chronic administration of carbon tetrachloride or of sodium selenate.

*Experimental.* A basal diet\* was adopted in which the amounts of methionine, choline and cystine, although relatively low, were adequate for good growth during the period of most rapid gain in body weight, and were sufficient to prevent the deposition of fat in the liver at this time. Eighty female rats,

weighing from 130 to 170 g, were divided into two principal groups. To one group carbon tetrachloride was given by stomach tube (0.2 cc of 20% solution in corn oil) 3 times weekly for a period of 8 weeks. Sodium selenate was added to the diet of the other group in an amount to make the selenium content 20 parts per million. Each of the principal groups was divided into 4 subgroups which received, respectively, the supplements shown in Table I. The animals were kept in individual cages and both food and water were offered *ad libitum*. Food intakes and body weights were recorded.

A protective effect of methionine was observed in the group fed selenium, but was not confirmed in a subsequent experiment of an apparently identical nature. The fact that this second set of diets was prepared less frequently than at our customary weekly intervals and stored without the usual refrigeration suggested that deterioration of a labile component might have occurred. A third experiment (Se3) was therefore performed in which the content of alpha-tocopherol in the diet was varied. Forty female rats weighing from 130 to 160 g were divided into 4 groups which were fed diets containing 20 p.p.m. of selenium. The first group received the same basal diet as was fed in the previous experiment except that no alpha-tocopherol was included. The supplements added to the other groups are shown in Table I. In other respects the procedure remained the same.

At the conclusion of the experiments autopsies and appropriate histological examinations were made. Liver lipids were estimated according to the method of Best, Lucas, Patterson and Ridout(1).

*Results.* 1. All the groups receiving carbon

\* Alcohol-extracted peanut flour 30, casein 2, fibrin 1, salts 4, beef dripping 15, corn oil 5, dextrin 10, cornstarch 10, vitamin powder 1, cod liver oil concentrate 0.015, alpha-tocopherol acetate 0.010, dl-methionine 0.130, choline chloride 0.250, sucrose to 100.

The vitamin powder was of such a composition that the intake per 10 g of diet was as follows: Biotin 3 γ, thiamine hydrochloride 50 γ, riboflavin 25 γ, pyridoxine hydrochloride 20 γ, calcium pantothenate 100 γ, nicotinic acid 100 γ, folic acid 5 γ, 2-methyl 1,4-naphthoquinone 10 γ, inositol 5 mg, para-amino benzoic acid 1 mg.

The cod liver oil concentrate supplied at least 300 i.u. vitamin A and at least 75 i.u. vitamin D per 10 g of diet.

1. Best, C. H., Lucas, C. C., Patterson, J. M., and Ridout, J. H., *Biochem. J.*, 1946, v40, 368.

TABLE I. Effect of Choline, Methionine, Cystine on Toxic Liver Damage.

Group	Supplement	Avg food intake per day (g)	Avg change body wt (g)	Lipid content % wet liver wt	Liver damage				Remarks
					Gross nodules	Necrosis or hemorrhage	Fibrosis		
<i>CCl<sub>4</sub></i>									
1	None (basal)	9.1	+31.3	9.32	++	+	++	++	Typical appearance of CCl <sub>4</sub> damage
2	0.15% choline chloride	9.7	+33.0	12.47	++	+	+++	+++	
3	0.5% DL-methionine	9.4	+23.0	12.58	++	+	++, +++	++	
4	0.4% cystine	9.7	+27.8	11.30	++	+	++		
Se 1									
1	None (basal)	6.9	+ 3.4	5.17	+	++, +++	+	+	
2	0.15% choline	7.4	+26.1	5.07	+	± to +++	+	+	3 of 10 normal grossly
3	0.5% DL-methionine	6.8	+ 8.1	5.06	—	— or ±	—	—	8 of 10 normal grossly
4	0.4% cystine	7.0	+11.5	5.14	+	++, +++	+	+	
Se 3									
1	None (basal)	6.2	— 5.1	—	±	++, +++	±	±	
2	0.05% alpha-tocopherol	6.6	—11.0	—	±	+ to +++	±	±	
3	0.5% methionine	7.7	— 4.4	—	±	+ to +++	±	±	
4	0.05% alpha-tocopherol 0.5% methionine	7.8	— 3.1	—	—	— to ±	—	—	7 of 10 normal grossly

tetrachloride showed a similar degree of hepatic injury. Fatty and hydropic degeneration, cellular necrosis, fibrosis, and regeneration of parenchymal cells were observed in almost every liver. None of the supplements appeared to protect this organ against damage. Food intakes, survival and gain in body weight were of a similar order in all groups.

2. A considerable degree of protection against damage to the liver was conferred by methionine in the rats that received selenium. The amount of damage did not differ very greatly in the other 3 groups. In the gross the livers were dark red with lighter hemorrhagic areas, often with a yellowish-white "fringe" at the edge of the lobes. Microscopically the type of injury consisted of mild degenerative changes of the parenchymal cells, dilatation of the sinusoids apparently originating in the midzone of the lobule, hemorrhage into the lobule with necrosis and subsequently organization(2). Milder cases (including a few in the methio-

nine-treated group) showed only the first one or two of these changes, while severe cases sometimes had large hemorrhages and extensive focal necrosis. Ascites was commonly associated with the more severely injured livers. Three livers from the group receiving choline showed little gross evidence of damage, although microscopically definite pathological changes existed. This group also showed the largest intake of food and greatest gain in body weight.

3. In a second experiment in which these treatments were repeated all 4 groups showed a similar degree of mild damage. In the third experiment (Se3), rats of the first three groups exhibited a similar degree of liver injury. In the fourth group (basal diet plus methionine plus alpha-tocopherol) little evidence of damage was present.

Results of the 3 experiments described are summarized in Table I.

2. Lillie, R. D., and Smith, M. I., *Am. J. Path.*, 1940, v16, 223.

**Discussion.** Previous work on the protective effects of lipotropic factors or substances related to them against hepatotoxins has usually been carried out using diets low in protein or other nutrients. Drill and Loomis(3) showed that no extra protection against liver damage due to carbon tetrachloride was given by methionine. This result has been confirmed in the present study. Choline or cystine seems to be equally ineffective. A protective action of methionine against selenium poisoning has been observed by several investigators, notably Smith(4) and Lewis, Schultz and Gortner(5). The latter workers reported that a diet containing 30% casein afforded significantly greater protection against selenium intoxication than did an equicaloric diet containing 6% casein, but that the addition of methionine to the 6% casein diet also afforded protection. They note that the effect was not always so marked with the methionine supplement as with the high protein diet. Cystine did not have the same beneficial effect as did methionine. In these experiments the addition of methionine corrected a deficiency of one essential amino acid, but other deficiencies still existed.

In our experiments the basal diet was not deficient according to current knowledge in any essential amino acid, and judged by the usual criteria was "adequate" in other respects. In spite of this, methionine produced a substantial protective effect. The apparent ineffectiveness of methionine in the second experiment led to the third experiment, in which it was shown that the protective reaction requires the presence of both alpha-tocopherol and methionine.

The significance of this finding is not apparent. Several papers have reported that massive necrosis of the liver, first believed to result from a single deficiency of methionine or of cystine(6-8), occurs only when an associated deficiency of alpha-tocopherol is

present(9,10). The apparent preventive action of alpha-tocopherol against hemoglobinuria produced by alloxan(11), and the protective effect of methionine, of cysteine or glutathione against alloxan diabetes(12-14) are further examples of reactions in which a possible relationship between the tocopherols and sulphhydryl compounds has been suggested(11). In the instance reported in this communication, as in the report cited(5), cystine did not have the protective effect of methionine. Is it possible that alpha tocopherol enables the potential -SH group of methionine to remain "free" and take part in a detoxification reaction, whereas in the case of cystine the potential -SH groups are less readily available? The observation that choline had little protective effect would appear to exclude the lipotropic activity of methionine as a factor of primary importance. However, an observation that may have a bearing on this is mentioned here because it is not our intention to do further work on selenium poisoning at the present time. The occurrence of 3 livers with only minimal damage in the group fed the choline supplement may indicate that further experimental work is desirable before excluding the lipotropic effect completely.

**Summary.** 1. The addition of supplementary methionine, choline or cystine to an "adequate" basal diet failed to protect the livers of rats against damage produced by the

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chronic oral administration of carbon tetrachloride. 2. A supplement of methionine, but not of cystine or of choline, showed a protective effect against damage produced by feeding sodium selenate (selenium 20 p.p.m.). 3. This action was demonstrated only in the presence of alpha-tocopherol. The relationship is discussed briefly.

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### Experimental Histoplasmosis. Susceptibility of Male DBA Line 1 Mice By Various Routes of Injection. (18120)

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It has recently been shown that, by intracerebral injection, male dba line 1 mice, 4-5 weeks of age, are uniformly susceptible to *Histoplasma capsulatum* and are apparently more so, by this route of injection, than several other strains(1,2). In the present report it will be shown that this strain of mice is more susceptible to infection with *Histoplasma* by this route of injection than by any other route.

**Procedure.** The mice used in these experiments were primarily dba line 1. In addition, a few Bar Harbor C 57 Black mice, lines 6 and 10, also were employed. All mice were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and were 4-5 weeks of age, weighing 10-18 g at time of injection. Altogether, in the experiments in which routes of injection were compared, 137 male and 60 female dba line 1 mice, 35 male and 34 female Bar Harbor C 57 Black, line 10, and 23 male and 23 female Bar Harbor C 57 Black mice, line 6 were injected with *Histoplasma*. In addition to the above animals, a few mice in each group were injected with saline alone to serve as controls. However, since it was found that, in general, male mice of both strains were more susceptible than females, the present

report will deal only with the results obtained with male animals. The strain of *Histoplasma*, No. 155, and the method of preparation of the inoculum were the same as that used in previous studies of this series(1). Three routes of injection were employed; for intracerebral injection, each mouse was injected with 0.02 ml of a given dilution of a saline suspension of the yeast phase of the fungus or 0.02 ml of saline alone; for intravenous injection, each animal was given 0.1 ml of the saline suspension of the fungus or 0.1 ml of saline alone; for intraperitoneal injection, each animal was given 0.5 ml of the saline suspension of the fungus or 0.5 ml of a suspension of the fungus in 5% mucin, or 0.5 ml of saline or mucin alone. The mucin suspension was prepared according to the method of Milzer and Levine(3). Equal parts of a 1-25 or a 1-50 saline suspension of the yeast phase of the fungus and the 5% mucin suspension were then mixed to give a 1-50 or a 1-100 dilution of the fungus in mucin. Serial dilutions were then prepared from this suspension with sterile physiological saline.

The estimation of the number of viable organisms actually injected, cultures of tissues obtained at autopsy, and other procedures followed were the same as those previously reported(1). A description of the gross lesions present at autopsy and the results of

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## EXPERIMENTAL HISTOPLASMOSIS

TABLE I. Results, in Terms of Mortality, Obtained in a Series of Experiments in Which Male dba Line 1 Mice Were Injected by Specified Routes with a Saline or Mucin Suspension of the Yeast Phase of a Single Strain of *Histoplasma capsulatum* at Different Dose Levels.

Exp. No.	Dilution employed	Route of injection							
		Intracerebral		Intravenous		Intraperitoneal		Organisms suspended in 5% mucin	
		Estimated dose per inj.*	No. of deaths†	Estimated dose per inj.*	No. of deaths†	Estimated dose per inj.*	No. of deaths†	Estimated dose per inj.*	No. of deaths†
I	1/100	319	3/3	3186	0/3	7965	3/3	13500	3/3
II	1/50	678	—	6774	1/5	16935	4/5	17500	5/5
	1/100	339	4/5	3387	1/5	8468	0/5	8750	2/5
	1/200	169	5/5	1694	1/5	4234	0/5	4375	1/4‡
	1/400	85	5/5	847	0/4	2117	0/5	2187	0/5
	1/800	42	5/5	423	—	1059	—	1094	—
	Saline	0	0/5	0	0/3	0	0/5	0§	0/5
IV	1/50	176	—	—	—	—	—	2400	2/6
	1/100	88	5/5	—	—	—	—	1200	—
	1/200	44	6/6	—	—	—	—	600	—
	1/400	22	6/6	—	—	—	—	300	—
	1/800	11	4/6	—	—	—	—	150	—
	1/1600	6	4/6	—	—	—	—	75	—
	1/3200	3	1/6	—	—	—	—	38	—
	1/6400	1	4/6	—	—	—	—	19	—
	Saline	0	0/4	—	—	—	—	0	—
Total No. of animals	Inj. with a suspension of <i>Histoplasma</i>	52/64	—	3/22	—	7/23	—	13/28	—
	Inj. with saline alone	0/9	—	0/3	—	0/5	—	0/5	—

\* Expressed in thousands of organisms.

† No. of mice which died spontaneously before the 30th day after injection.

‡ One additional animal is not included in the tabulation as it died on the 4th day after injection of a generalized peritonitis, the result of traumatic perforation of the intestine.

§ Injected with 5% mucin alone.

the microscopic examination of sections from all tissues taken at autopsy will be presented later.

**Results.** The results, in terms of mortality obtained, of the inoculation of male dba line 1 mice by each of the three routes employed are presented in Table I.

A comparison of the death rates obtained immediately reveals sharp differences, depending on the route of inoculation. Pooling the results of all experiments for a single route of injection, it can be seen that an intracerebral injection, for example, of 1000 to 340,000 organisms, resulted in the spontaneous death, within 30 days after injection, of 52 of 64, or 81.3%, of the mice injected by this route. Intraperitoneal injection, however, of 2,000,-

000 to 17,000,000 organisms, suspended in saline, resulted in the death of only 7 of 23, or 30.4%, within the same interval. The addition of mucin for intraperitoneal injection, within the same dose range, increased the percentage of spontaneous deaths to 46.4% (13 of 28).

Not only were marked differences observed in the per cent of mortality obtained, depending on the route of injection, but also the time by which any given percentage of the animals were dead varied with the route (Fig. 1). By the fourteenth day following intracerebral injection, 47 of 64, or 73.4%, of the mice were dead; by intraperitoneal injection in saline only 2 of 23, or 8.7%; and, by intraperitoneal injection in mucin, only

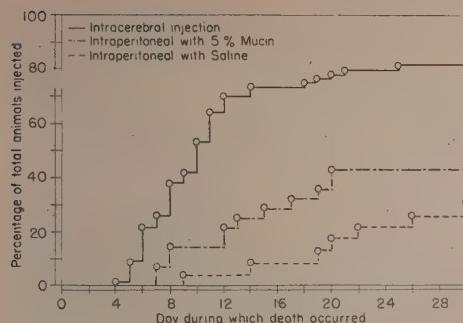


FIG. 1.

Cumulative death rates observed among male dba line 1 mice within 30 days of injection with varying doses of the yeast phase of *Histoplasma capsulatum*.

7 of 28, or 25.0%, were dead. Similar results were obtained with the Bar Harbor C 57 Black mice. For example, 9 of 11, or 81.8%, of line 6 died following intracerebral injection of 44,000 to 350,000 organisms, while only 5 of 12, or 41.7%, died following injection with 4,000,000 to 8,000,000 organisms suspended in mucin. The same doses resulted in the death of 9 of 14, or 64.3%, of line 10 mice following intracerebral injection, and 7 of 12, or 58.3%, following intraperitoneal injection in mucin. Although in the latter group almost as high a percentage were killed by intraperitoneal injection in mucin as by the intracerebral route, the dosage necessary to produce this fatality rate, by intraperitoneal injection, was twenty to almost one hundred times that given intracerebrally. No control animal in any experiment died before the sacrifice date.

*Histoplasma* was recovered in culture from the brain and/or spleen of each mouse injected with the fungus in these experiments except 8. Of these 8, cultures from 6 were overgrown with contaminants. In one additional animal, the tissues were so badly autolyzed at autopsy that no cultures were made. The remaining animal was a dba line 1 mouse injected intraperitoneally with mucin.

*Comment.* The finding that the addition of mucin for intraperitoneal injection of the yeast phase of *Histoplasma* into mice increases the susceptibility of these animals to this fungus is in agreement with the recent report of Campbell and Saslaw(4). However,

with the strains of *Histoplasma* employed by these authors, and with the strain used in this study, enormous doses of the fungus were required to produce a relatively high degree of mortality. Campbell and Saslaw, for example, found that an intraperitoneal dose of approximately 3,500,000 organisms of their Strain No. G-8 suspended in mucin were necessary to produce 66.6% to 85% mortality by the thirtieth day after injection, using White Swiss mice (Bagg strain). This same strain of *Histoplasma*, designated by us as No. 167, in doses of 7,000 to 115,000 organisms, by intracerebral injection, killed 26 of 30, or 86.6%, of male dba line 1 mice within thirty days after injection(5).

It has also been shown that dba line 1 mice are more susceptible to *Histoplasma* than White Swiss mice and that the time of death, after intracerebral injection, can be regulated by the dosage employed(1,2). Therefore, it would seem, from the data presented above, and from a comparison of the results obtained by Campbell and Saslaw (4) with that obtained by us in other experiments, that mice are much more susceptible to intracerebral injection with *Histoplasma* than to intraperitoneal injection in saline or mucin.

*Summary.* In a series of experiments it has been shown that male dba line 1 mice are relatively resistant to intravenous injection of the yeast phase of *Histoplasma capsulatum*. Intraperitoneal injection of enormous numbers of the organisms suspended in saline produced occasional deaths. Intraperitoneal injection of approximately the same numbers of viable organisms suspended in 5% mucin increased the death rate. Intracerebral injection, however, was far superior to any of the other routes employed, both with respect to the numbers of organisms necessary to produce death 30 days after injection and the percentage of fatalities obtained within this period of time.

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## Distribution of Total Ferritin in Intestine and Mesenteric Lymph Nodes of Horses After Iron Feeding.\* (18121)

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It has been postulated that a mechanism exists whereby the gastrointestinal mucosa regulates the amount of iron absorbed from the intestine. Hahn *et al.*(1) and Granick(2) suggested that the ferritin and apoferitin of the gastrointestinal mucosa are concerned with the absorption of iron. The protein apoferitin, which can combine with ferric iron to form ferritin, was thought to accumulate absorbed iron until saturated, whereupon the establishment of an equilibrium state between the ferric iron in ferritin and ferrous iron prevented further absorption of iron from the intestine. This suggestion was apparently supported by experiments conducted by Granick(3) whose semi-quantitative data indicated that only traces of ferritin are present in the duodenal mucosa of normal guinea pigs, but that after feeding iron, the ferritin content of the duodenal mucosa increases rapidly, and then falls gradually, finally reaching the level of control animals 3 to 6 days after feeding the iron. Prevention of further absorption of iron from the intestine when the apoferitin is saturated with ferric iron has been termed "mucosal block."

Gillman and Ivy(4) presented histochemical

\* Material taken from a thesis submitted by B. W. Gabrio to the University of Rochester in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry. This paper is based on work performed under contract with the U. S. Atomic Energy Commission at the University of Rochester Atomic Energy Project. The authors wish to express their appreciation to Professor Elmer Stotz and Dr. A. L. Dounce for their interest in this investigation.

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evidence indicating a progressive increase in the amount of iron in the intestinal mucosa and mesenteric lymph glands after feeding iron, but were unable to demonstrate consistently an increase in crystallizable ferritin in the intestine of the guinea pig after feeding iron, as had been reported by Granick. However, Granick's result has been reproduced in this laboratory.<sup>†</sup>

The experiments to be reported in this paper deal primarily with the determination of total ferritin<sup>‡</sup> in the intestine and mesenteric lymph nodes of the horse by an immunochemical technic, following the ingestion of iron. We were interested in discovering whether ferritin is involved in the absorption of iron in the intestine of the horse, an animal from which ferritin can be obtained with ease and in quantity; and also in determining whether the increase in the iron content of the mesenteric lymph nodes after the ingestion of iron might be attributable to ferritin.

*Experimental procedure.* Three 8-year-old male horses were used in the experiments which follow. All three animals eventually were sacrificed by being shot through the head. Horse No. 1 which served as the control, was fasted for about 18 hours before being sacrificed, while horse No. 2 and horse No. 3, after an 18 hour fast, were fed gelatin-coated capsules of ferrous ammonium sulfate (containing a total of 30 g of iron) with the use of a capsule gun. Horse No. 2 was sacrificed 24 hours after the administration of iron, while Horse No. 3 was sacrificed 48 hours after the feeding of iron. The following tissue samples were taken from each horse for analysis: 3 successive portions of intestine beginning just below the pyloric sphincter,

<sup>†</sup> Gabrio, B. W., unpublished work.

<sup>‡</sup> Hereafter, unless specified otherwise, the term, ferritin as applied to these experiments, will refer to total ferritin, which includes ferritin, apoferitin, and intermediate forms.

2.5 feet, 5 feet, and 4 feet in length; the last 3 feet of intestine; and the mesenteric lymph nodes. Each sample was washed with isotonic saline.

Extracts of ferritin were prepared from the tissue to be assayed by grinding the tissue in a Waring blender with 2 times its weight of water. The suspension was heated to 75-80°C and the coagulum was centrifuged and discarded. Sufficient acetic acid (50% by volume) was then added with stirring to adjust the pH to 4.6-4.7, and the solution was allowed to stand for 2 hours at room temperature(5). Any precipitate which formed was centrifuged and discarded. The ferritin was then precipitated by adding 35 g of  $(\text{NH}_4)_2\text{SO}_4$  per 100 ml of solution, and after approximately 8 hours, the precipitate was centrifuged down and dissolved in a small amount of water. The solution was dialyzed against isotonic saline, and the clear dialyzed solution, containing ferritin, was used in the quantitative test described in the following paragraph. The procedure described in the above paragraph is based upon the work of Granick(6). The amounts of ferritin in the tissue extracts were determined quantitatively by an immunochemical technic, which was essentially the same as that described by Mazur and Shorr(5). This technic has been worked out independently in our laboratory<sup>§</sup> but had not been published by us. In this method, the antigenic properties of ferritin are utilized in the following way. Horse spleen ferritin prepared according to the procedure of Mazur and Shorr(5) with slight modifications<sup>||</sup> is injected into rabbits in increasing amounts over a 4-week period. A total of 4.5 mg of antigen nitrogen is thus injected into each rabbit. The rabbits are bled 5 days after the last injection and the antisera are collected. The antigen-antibody reaction is measured by the precipitin test(8).

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|| Mazur, A., personal communication.

The tissue extract is reacted with the horse spleen ferritin antiserum, and the total nitrogen content of the specific precipitate is determined by the micro-Kjeldahl method. The amount of total ferritin nitrogen present is read from a standard curve prepared with known amounts of ferritin. In our work, the colors of the specific precipitates were noted, since it has been shown(7) that the iron is precipitated quantitatively with the antigen.

*Results and discussion.* Since the antibody produced during the process of immunization with ferritin reacts with the protein moiety of ferritin, and since both apoferritin and ferritin react identically with the antiserum, the determinations made in this investigation measure total ferritin. The latter term includes apoferritin, iron-saturated ferritin, and any ferritin which may be only partially saturated with respect to iron. Since the colors of the specific precipitates obtained with the tissue extracts of the control horse No. 1 were paler in color than those obtained from the iron-fed horses, it is likely that in the tissues of the control horse the ratio of ferritin to apoferritin was very low, or else that a ferritin only partially saturated in iron was being precipitated.

The procedure used in this study for extracting and precipitating ferritin has not been proved quantitative over the entire range of ferritin concentrations studied, but it was the only method recorded in the literature(6) at the time this work was carried out. More recently, the procedure has been modified by Mazur and Shorr(7).

The distribution of total ferritin in the intestine and mesenteric lymph nodes of the untreated horse (No. 1) and of the horses fed iron (No. 2 and 3) is presented in Table I. (A very small amount of undissolved ferrous ammonium sulfate was found in the stomachs of the latter two animals upon sacrifice.) In the control horse (No. 1) ferritin was found to occur in the first 2.5 feet of

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## DISTRIBUTION OF FERRITIN IN HORSES

TABLE I. Distribution of Ferritin in Intestine and Mesenteric Lymph Nodes of Horses After the Ingestion of Iron.

Tissue analyzed	Horse No. 1 (fasted control)	Total ferritin nitrogen, $\mu\text{g}$ per foot of intestine, or $\mu\text{g}$ per g wet mesenteric lymph node	
		Horse No. 2 (fasted, then sacrificed 24 hr after feeding ferrous ammonium sulfate 30 g Fe)	Horse No. 3 (fasted, then sacrificed 48 hr after feeding ferrous ammonium sulfate 30 g Fe)
1st 2.5 ft of intestine beginning at pylorus	351	4225	2592
Next 5 ft of intestine	132	320	1546
" 4 ft "	131	79	1717
Last 3 ft "	205	270	783
Mesenteric lymph nodes	15	57	77

intestine beginning just below the pyloric sphincter, and to a lesser extent in the following 5 foot and 4 foot lengths of intestine, as well as in the last three feet. The presence of a small amount of ferritin was also noted in the control animal (No. 1) in the mesenteric lymph nodes.

The increase in the total ferritin content of the intestine of the horse after the feeding of iron indicates that ferritin probably is involved in the phenomenon of iron absorption in this animal. It appears that during the first 24 hours following the administration of iron, the absorption of the iron has occurred largely through the first 2.5 feet of intestine, since a profound increase in the amount of total ferritin in this portion of the intestine during the first 24 hour period has been demonstrated. However, during a 48 hour interval after feeding of iron, the iron was absorbed in the first 11.5 feet of intestine and also in the last 3 feet. Essentially then, these results are similar to those obtained by Granick(3) using the guinea pig, and that portion of the process of iron absorption which involves ferritin appears to be comparable in the two species.

Since there was a notable increase in the amount of total ferritin in the mesenteric lymph nodes of both horses No. 2 and 3 after the feeding of iron, the increase in histologically-demonstrable iron in the mesenteric lymph nodes following the ingestion of iron reported by Gillman and Ivy(4) may be attributable to an increased ferritin content

of this tissue. Thus the results reported in this paper suggest that the lymphatic system is involved in iron absorption and that ferritin assumes a rôle in this case also.

**Summary.** 1. The distribution of total ferritin in the intestine and mesenteric lymph nodes in a control horse was compared with the corresponding distributions in 2 horses fed iron, using an immunochemical technic for determining the ferritin in extracts of the tissues in question.

2. Ferritin was found to be present in the first 11.5 feet of intestine of the control horse as well as in the last 3 feet of intestine. Small amounts of ferritin were detected in the mesenteric lymph nodes of the control animal.

3. Twenty-four hours after the oral administration of an amount of ferrous ammonium sulfate containing 30 g of iron to a second horse the total ferritin content was appreciably increased in the first 2.5 feet of intestine while 48 hours after feeding the same amount of ferrous ammonium sulfate to a third horse, there were notable increases in the amounts of total ferritin in the first 11.5 feet and in the last 3 feet of intestine.

4. Twenty-four hours after feeding iron to a second horse there was approximately a 4-fold increase in the amount of total ferritin in the mesenteric lymph nodes based on the value for the control horse, while 48 hours after feeding iron to the third horse, more than a 5-fold increase was noted, as judged by the value for the control horse.

5. The data indicate that ferritin is involved in the phenomenon of iron absorption through the intestine of the horse, and that the lymphatic system is concerned with iron absorption by some process in which ferritin also plays a rôle.

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### Collagen Content of Guinea Pig Tissues. (18122)

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A chemical method has been described by which collagen, the major fibrous component of connective tissue, can be measured quantitatively in tissues(1). This technic has been used to study the effects of physiologic and pathologic processes on the formation, distribution and destruction of collagen(1-13). During the course of an investigation of the effect of ascorbic acid deficiency on the collagen content of guinea pig tissues(6), the necessity for a more detailed study of the

normal pattern of connective tissue distribution in the body became more evident. Therefore, this study was undertaken.

**Experimental.** One hundred and eight male guinea pigs weighing between 60 and 1000 g were selected from an inbred AMC strain at this laboratory. The animals were maintained on a diet of Derwood Rabbit Chow pellets and fresh greens daily *ad libitum*. They were weaned at approximately 200 g body weight. The guinea pigs were sacrificed with a blow to the back of the skull, which occasioned a variable amount of bleeding

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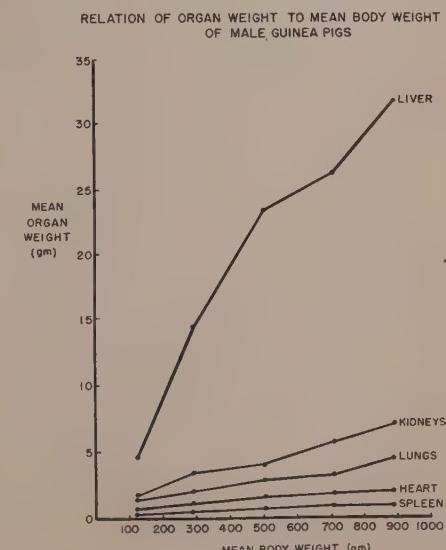


FIG. 1.

## TISSUE COLLAGEN CONTENT

TABLE I. Collagen Content of Wet Tissues.

Group (g)	No. of animals	Mean wt (g)*	Lungs (%)*	Liver (%)*	Kidneys (%)*	Spleen (%)*	Heart (%)*	Skeletal muscle (%)*
60-199	11	121 ± 14	1.01 ± .10	.36 ± .06	.47 ± .06	1.21 ± .32	.80 ± .13	.96 ± .12
200-399	17	296 ± 13	1.45 ± .08	.63 ± .08	.62 ± .05	.74 ± .08	1.06 ± .06	.89 ± .04
400-599	10	501 ± 19	1.47 ± .09	.47 ± .07	.60 ± .05	.54 ± .07	1.18 ± .05	1.00 ± .08
600-799	10	706 ± 13	1.79 ± .12	.39 ± .03	.56 ± .03	.55 ± .08	1.04 ± .05	.98 ± .06
800-999	10	888 ± 13	1.67 ± .12	.37 ± .04	.61 ± .04	.50 ± .05	1.06 ± .06	1.25 ± .09

\* Includes stand. error of the mean.

from the cranial orifices. They were weighed and dissected immediately. Samples of skeletal muscle, heart, lungs, liver, kidneys and spleen were removed in a manner described previously(6). In 58 animals, quantitative chemical measurements of the collagen content of the various tissues were made according to the method of Lowry *et al.*(1). If the weight of the individual organ was 4 g or less, the entire organ was used for the chemical analysis. In all instances the heart and spleen were treated in this manner. If the organ was larger than 4 g, it was weighed to the nearest 0.1 g and a sample taken for the chemical procedure. Liver and skeletal muscle fell into this category. Depending on the weights of the lungs and kidneys, analyses were conducted either on the single organ or on the pair of organs. The proportion of water in the tissues of the remaining guinea pigs was measured. Approximately 1 g of tissue was minced, weighed and desiccated to constant weight.

The relation of mean organ weight to mean body weight is illustrated in Fig. 1. The 58 animals were divided by weight into 5 groups. The rate of growth was characteristic for each organ, the liver growing most rapidly and the spleen least rapidly.

The collagen content of the various tissues on a wet weight basis is listed in Table I. The amount of tissue collagen varied with the type and size of the organ. The lungs contained the largest, and the liver the smallest concentration of collagen. The changes of collagen content with age were not uniform in all the tissues. One pattern was exemplified by the lung, in which a progressive increase occurred with increase of body weight. The proportion of connective tissue

in skeletal muscle remained approximately constant except for a significant increase in the oldest group. On the other hand, there was an initial rise of the concentration of collagen in the kidneys and heart, after which the collagen level remained unaltered. In the liver the proportion of collagen increased at first, attained a maximum and then decreased. The spleen exhibited the most unusual change with age; the concentration of collagen decreased progressively as the animal increased in size. When values for collagen content of wet tissues were corrected for water content, as obtained from 50 guinea pigs of comparable weight, the derived data of Table II were calculated. Expression of collagen on a dry weight basis did not alter significantly the findings described above. The absolute amounts of collagen in the tissues are diagrammed in Fig. 2. The values are the product of the organ weight and the collagen concentration. There was an increase of the amount of collagen in each organ with somatic growth, despite the fluctuations of the proportions of collagen in the tissue.

*Discussion.* Several factors may be considered in evaluating these results. For a population of healthy guinea pigs, body

TABLE II. Collagen Content of Dry Tissues.

Group (g)	Lungs (%)	Liver (%)	Kidneys (%)	Spleen (%)	Heart (%)	Skeletal muscle (%)
60-199	5.05	1.21	2.41	5.53	3.94	4.44
200-399	7.59	2.23	2.97	3.46	5.58	4.16
400-599	7.42	1.64	2.83	2.51	6.21	4.57
600-799	8.91	1.32	2.55	2.49	5.42	4.28
800-999	8.15	1.28	2.81	2.25	5.44	5.46

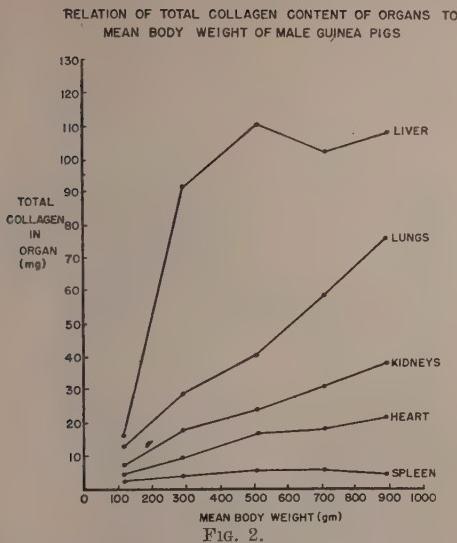


FIG. 2.

weight has been satisfactorily used as the criterion of age(14). In this investigation the effect of sex was not explored; only male animals were studied. It was not possible to determine simultaneously both collagen and water content of the tissues in the same animals. Therefore, 2 sets of data were obtained and pooled in order to calculate the collagen concentrations on a dry weight basis. Although it would have been more desirable to have the data from the same animals, these results are nevertheless valid. The other constituents of the tissues were not measured chemically. Relative changes in the proportions of connective tissue may be occasioned by variations of the other constituents. As growth proceeds, there is increased accumulation of connective tissue in the different organs.

The amount of collagen in guinea pig liver, kidney, skeletal muscle and spleen does not

differ greatly from the corresponding organs of the human and rat(1,3-5,7,9). The values for the guinea pig heart could not be compared with reported values for rat(9) or human(12). In our study, the entire organ, including valves, chordae tendineae and auricles were examined *in toto*; in the other studies, only the cardiac musculature was examined. No previously published values for the normal collagen content of lung have been found.

The general statement that progressive fibrosis of tissues occurs with aging should be qualified. In these experiments, older tissues contained more collagen than younger ones, with the exception of the spleen. Lowry *et al.*(7) failed to find a correlation between the age and the connective tissue content of rat livers. In contrast, there was a definite alteration of the liver collagen content with age in the guinea pig. Increased fibrosis of rat skeletal muscle(9) and renal cortex(7) in older animals has been reported. Our findings confirmed the former but failed to substantiate the latter in the guinea pig whole kidney.

**Summary.** 1. Chemical measurements were made of the collagen content of the lungs, liver, kidneys, spleen, heart and skeletal muscle of 58 guinea pigs weighing between 60 and 1000 g. 2. The concentration of connective tissue components varied with different tissues; the lungs contained the greatest proportion, and the liver the least proportion, of collagen. 3. Tissues from older animals usually contained a higher percent collagen than comparable younger tissues, except for the spleen where the converse was true. 4. The values obtained for guinea pig tissues were in essential agreement with previously reported figures for other species.

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## Effect of Sulfasuxidine on the Interrelation of Folic Acid, Vitamin B<sub>12</sub> and Vitamin C.\* (18123)

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In a previous communication(1), we reported that the addition of vitamin C and vitamin B<sub>12</sub> to semi-purified rations stimulated chick growth and the *in vivo* synthesis of folic acid (PGA) as measured by liver storage. We also reported that PGA produced an increase in the amount of vitamin B<sub>12</sub> stored in the liver. We suggested that the effects noted were due, particularly in the case of vitamin C, to a stimulation of intestinal synthesis. It is the purpose of this paper to report further studies on this problem.

**Experimental.** Straight run (New Hampshire ♂♂ X Single Comb White Leghorns ♀♀) crossbred chicks, which were the progeny of hens fed diet B-1 described previously(2), were used in all studies. The chicks were housed in electrically heated batteries with raised screen floors. Feed and water were supplied *ad libitum*. The chicks were wing banded and weighed at one day of age. Weights were recorded at weekly intervals.

All chicks were placed on a PGA-deficient

ration containing sucrose 61 g, alcohol extracted casein 18 g, gelatin 10 g, salts V(3) 6 g, soybean oil 5 g, L-cystine 0.3 g, thiamine hydrochloride 0.3 mg, riboflavin 0.6 mg, nicotinic acid 5.0 mg, pyridoxine hydrochloride 0.4 mg, calcium pantothenate 2.0 mg, choline chloride 150 mg, biotin 0.03 mg, inositol 100 mg, 2-methyl-1, 4-naphthoquinone 0.05 mg, and  $\alpha$ -tocopherol 0.3 mg. Fortified haliver oil (60,000 U.S.P. units of vitamin A, 6,000 U.S.P. units of vitamin D<sub>3</sub> per g) was given by dropper (2 drops per bird per week).

In all cases, groups of 12 chicks were used throughout except in Exp. 3 where groups of 10 birds were used. Crystalline vitamin B<sub>12</sub> was injected into the pectoral muscle with a 1 ml calibrated syringe. At the termination of the depletion period, which in all cases was 2 weeks, the chicks were sorted and divided into groups according to weight and percent gain. A test period of 2 weeks followed, at the termination of which the birds were sacrificed, and the livers removed, frozen immediately, and stored at freezing temperatures until assayed. PGA and vitamin B<sub>12</sub> were extracted as described previously(1) except that one-half the amount of trypsin originally called for was used. PGA was measured microbiologically with *Streptococcus faecalis* R as a test organism using the medium of Luckey *et al.*(4). Vitamin B<sub>12</sub> was measured similarly with *Lactobacillus leichmannii* ATCC 4797 using the medium of Skeggs *et al.*(5), modified by Thompson *et al.*(6).

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TABLE I. Effect of Sulfasuxidine on Chick Growth, Liver Storage, and Caecal Content of PGA and Vitamin B<sub>12</sub>.\* Exp. 1.

Supplement	Wt during gain test, g	Liver storage				Caecal contents	
		PGA		Vit. B <sub>12</sub>		PGA γ/g dried wt	Vit. B <sub>12</sub> γ/g dried wt
		γ/g	Storage equivalent†	γ/g	Storage equivalent†		
50 γ PGA/100 g of ration	40	1.17	3.90 ± 0.39	0.04	0.05 ± 0.00	2.65 ± 0.25	4.50 ± 0.37
50 γ PGA/100 g of ration + vit. B <sub>12</sub> (0.1 γ/day)‡	65	1.04	3.90 ± 0.59	0.07	0.17 ± 0.01	9.57 ± 0.45	6.25 ± 0.34
50 γ PGA/100 g of ration + vit. C (100 mg %)	42	2.42	6.01 ± 0.69	0.02	0.06 ± 0.00	11.77 ± 2.11	5.20 ± 0.68
50 γ PGA/100 g of ration + sulfasuxidine (1%)	57	1.38	5.42 ± 0.87	0.09	0.12 ± 0.05	0.00 ± 0.00	4.30 ± 0.41
50 γ PGA/100 g of ration + sulfasuxidine (1%) + vit. B <sub>12</sub> (0.1 γ/day)‡	59	1.69	4.44 ± 0.56	0.12	0.24 ± 0.05	0.00 ± 0.00	5.10 ± 0.62
50 γ PGA/100 g of ration + sulfasuxidine (1%) + vit. C (100 mg %)	56	1.34	5.18 ± 0.62	0.01	0.06 ± 0.02	0.88 ± 0.27	3.42 ± 0.24

\* Eight observations/group including standard error

$$\sqrt{\frac{\sum d^2}{n(n-1)}}$$

† Storage equivalent =  $\frac{\gamma/\text{liver}}{\text{body wt}} \times 100$ .

‡ Injected.

*Results and discussion.* The addition of vitamin C or vitamin B<sub>12</sub> produced a small growth response. The addition of sulfasuxidine at the level of 1% also produced a growth response which was not increased when vitamin C or vitamin B<sub>12</sub> was added to the ration.

In studying the interrelation of vitamins of the B-complex through liver storage, one is limited by the inefficient manner in which the water soluble vitamins are stored in this as well as other tissues. Evaluation is further complicated by the difference in the total weights of the chicks receiving the different rations. This difficulty can be compensated for to some extent by reducing all values to a common base, namely, γ per liver per 100 g of body weight (storage equivalent). Our results (see tables) are presented as both γ per g liver and liver storage equivalent.

The results obtained upon the analysis of the livers of these chicks for PGA and vitamin B<sub>12</sub> were in accordance with previous work, namely, the addition of vitamin C resulted in a definite increase in the PGA stored in the liver. Such a stimulation was

not observed when sulfasuxidine was added to the ration. Caecal analysis of these groups (Table I) proved far more striking. The administration of vitamin B<sub>12</sub>, 0.1 γ per day by injection, produced a definite increase in the caecal level of PGA. The addition of vitamin C also increased the caecal PGA. The increase in caecal level of PGA upon administration of vitamin B<sub>12</sub> was as great as that observed upon the addition of vitamin C. There may be several explanations for the observed results. First, the flora in the caecum may differ from that in the areas of the tract where the major part of absorption occurs. However, work by Shapiro *et al.*(7) appears to rule this out since evaluation of their data shows that the normal chick, at the age of 4 weeks, has the same ratio of different types of flora regardless of the section of the tract analyzed. Secondly, vitamin B<sub>12</sub> may interfere with the absorption of PGA. If this were the case, one would expect an increase in the caecal PGA upon

## EFFECT OF SULFASUXIDINE

TABLE II. Effect of Sulfasuxidine on Chick Growth and Liver Storage of PGA and Vitamin B<sub>12</sub> in Presence of Excess Vitamin B.\*

Exp.	Supplement	Wt gain during test, g	PGA		Vit. B <sub>12</sub>	
			γ/g	Storage equivalent†	γ/g	Storage equivalent†
2	None	10	1.09	4.51 ± 0.92	0.02	0.08 ± 0.00
	Vitamin B <sub>12</sub> (0.1 γ/day)‡	27	0.79	3.12 ± 0.53	0.07	0.24 ± 0.00
	Vitamin C (100 mg %)	17	1.48	7.32 ± 0.53	0.03	0.08 ± 0.02
	Sulfasuxidine (1%)	11	1.01	3.28 ± 0.46	0.02	0.08 ± 0.00
	Sulfasuxidine (1%) + vit. B <sub>12</sub> (0.1 γ/day)‡	21	0.74	2.48 ± 0.38	0.15	0.41 ± 0.13
	Sulfasuxidine (1%) + vit. C (100 mg %)	11	1.64	6.14 ± 1.58	0.01	0.05 ± 0.00
	50 γ PGA/100 g of ration	74	1.17	4.00 ± 0.68	0.02	0.07 ± 0.00
	50 γ PGA/100 g of ration + vit. B <sub>12</sub> (0.1 γ/day)‡	111	1.18	5.60 ± 0.65	0.08	0.24 ± 0.04
	50 γ PGA/100 g of ration + vit. C (100 mg %)	106	1.95	8.85 ± 1.37	0.05	0.14 ± 0.03
	50 γ PGA/100 g of ration + sulfasuxidine (1%)	73	1.56	6.32 ± 0.85	0.03	0.07 ± 0.04
	50 γ PGA/100 g of ration + sulfasuxidine (1%) + vit. B <sub>12</sub> (0.1 γ/day)‡	113	2.13	6.83 ± 0.65	0.06	0.17 ± 0.04
	50 γ PGA/100 g of ration + sulfasuxidine (1%) + vit. C (100 mg %)	73	1.20	4.18 ± 0.76	0.01	0.06 ± 0.00

\* Eight observations/group including standard error

$$\sqrt{\frac{\sum d^2}{n(n-1)}}$$

† Storage equivalent =  $\frac{\gamma/\text{liver}}{\text{body wt}} \times 100$ .

‡ Injected.

the administration of vitamin B<sub>12</sub> and sulfasuxidine. This was not observed. Another possibility is that vitamin B<sub>12</sub> either inhibits the storage of PGA, aids in, or is the agent in the conversion and/or storage of a PGA like substance that lacks microbiological activity for *S. faecalis*.

Administration of sulfasuxidine, in all cases, greatly lowered the caecal level of PGA. This is in confirmation of previous work (8,9). This effect was observed regardless of whether vitamin C or vitamin B<sub>12</sub> were present. On the other hand, the administration of sulfasuxidine had no effect on the caecal level of vitamin B<sub>12</sub>.

Exp. 2 (Table II) was similar to Exp. 1 except that in this case the level of B vitamins in the ration was doubled in order to

compensate for any other B vitamin, besides PGA, that might be made limiting by the administration of sulfasuxidine. The results are essentially the same as in Exp. 1 except that a growth stimulation was observed upon the addition of vitamin B<sub>12</sub> and sulfasuxidine showing that sulfasuxidine does not inhibit the growth response attributed to vitamin B<sub>12</sub> if the level of the other B vitamins is adequate.

Another interesting phenomena is one that has been noted many times in this laboratory, namely the inclusion of sulfasuxidine in the ration, produces variable results. The effect of sulfasuxidine on the intestinal flora and the, in turn, effect on vitamin synthesis is well known. Consequently, if a new born chick receives an inoculum high in organisms that synthesize necessary vitamins, the inclusion of sulfasuxidine in the diet will result in a growth depression. On the other hand, if the inoculum received is high in organisms that have a high requirement for the necessary vitamins,

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TABLE III. Effect of Injected PGA and Vitamin C on Chick Growth, Liver Storage, and Caecal Content of PGA and Vitamin B<sub>12</sub>.\*

Exp.	Supplement	Wt gain during test, g	Liver storage				Caecal contents	
			PGA		Vit. B <sub>12</sub>		PGA γ/g dried wt	Vit. B <sub>12</sub> γ/g dried wt
			γ/g	Storage equivalent†	γ/g	Storage equivalent†		
3	None	20	0.98	3.22 ± 0.43	0.01	0.01 ± 0.00	4.71 ± 0.82	2.50 ± 0.22
	PGA (20 γ/day)	134	2.31	5.95 ± 0.77	0.07	0.16 ± 0.00	11.10 ± 0.67	4.70 ± 0.62
	Vit. C (25 mg/day)	20	1.27	3.40 ± 0.73	0.04	0.09 ± 0.10	4.25 ± 0.52	3.60 ± 0.54

\* Six observations/group including standard error

$$\sqrt{\frac{\sum d^2}{n(n-1)}}$$

† Storage equivalent =  $\frac{\gamma/\text{liver}}{\text{body wt}} \times 100$ .

the inclusion of sulfasuxidine in the diet will result in a growth stimulation since more of the vitamin injected by the animal would be available for absorption. Shapiro(7), has shown that in the case of the normal chick, on a natural ration, the predominant flora in the tract are lactobacilli. The high vitamin requirements of the lactobacilli are well known. If this is also the case with a sucrose semi-purified ration, the generally observed growth stimulation resulting upon the inclusion of sulfasuxidine in the diet is readily explained. Such a concept is further borne out by the observation that in Exp. 2 no change in growth occurred when sulfasuxidine was included in the ration.

In Exp. 3 (Table III) the injection of PGA at the level of 20 γ per day resulted in a definite growth stimulation and increase in the PGA stored in the liver. A marked increase in the liver storage of vitamin B<sub>12</sub> was also observed. The injection of vitamin C at the level of 25 mg per day had no effect on growth, or liver storage of PGA and vitamin B<sub>12</sub>. Analysis of the caecal contents were in correlation with the liver analysis. The injection of PGA resulted in a higher caecal

level of this vitamin, showing that PGA may be excreted into the tract under the proper conditions.

*Summary.* The administration of sulfasuxidine eliminates the growth response produced by vitamin C in the chick, on a semi-purified ration. No effect is observed on the growth response produced by vitamin B<sub>12</sub>, if the levels of the other B vitamins are adequate.

The administration of sulfasuxidine to a semi-purified ration eliminates the increase of the PGA liver storage produced by vitamin C. Both vitamin C and vitamin B<sub>12</sub> raise the caecal concentration of PGA in the chick. The administration of sulfasuxidine lowers the caecal levels of PGA to a minimum in all cases. Sulfasuxidine has no effect on the caecal level of vitamin B<sub>12</sub> in the chick.

Vitamin C, when injected, has no effect on growth, liver storage or caecal concentration of PGA in the chick. The injection of PGA markedly increased the liver storage and caecal levels of both PGA and vitamin B<sub>12</sub>.

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## Electron Microscopic Observations of Stromatolysis in Human Erythrocytes. (18124)

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(Introduced by O. H. Gaebler.)

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For an electron microscope study of *Treponema carateum*, preparations were made of tissue fluid from the skin lesions of a typical case of pinta. These preparations, not subjected to chemical fixation, showed a number of well-preserved erythrocytes and relatively abundant images which are thought to correspond to disintegrating, hemolyzed erythrocytes. The processes used to separate the treponemas from the other material present in the skin tissue fluid resulted in the hemolysis of most of the erythrocytes. The specimens were centrifuged in both normal saline and distilled water suspension for 30 minutes, respectively. A small portion of one tissue fluid batch was examined without centrifugal concentration and no distilled water was used in this case. The technic has been described in more detail elsewhere(1).

*Observations.* While no changes were observed in some cells, widely different degrees of hemolysis were observed in others, and some of the erythrocytes showed changes in cell surface configuration. A comparison of these latter images with those reported by several workers(2-10) using the optical microscope under controlled conditions of stro-

matolysis shows striking similarities. Further evidence of the stromatolytic nature of the observed changes is obtained by noticing appearances which are either identical or closely similar to the different stages of lysis of erythrocyte stroma reported by numerous workers. There is no basic difference in morphology, except for the superior image detail, between the electron micrographic appearances reported here and the photomicrographic ones depicted elsewhere. This is probably due in part to the absence of chemical fixation which is in accordance with previous findings(11).

Some images (Fig. 1 and 7) undoubtedly correspond to erythrocytes in relatively good condition, while others (Fig. 4, 14 and 15) are apparently erythrocytes which have dried flat to the supporting film in the usual processes of electron microscopy after suffering crenation and hemolysis. This interpretation also applies to other images such as those shown in Fig. 5, 11, 12 and 16, although they are often so distorted that they might be confused with dried liquid residues wherein the flow processes provoked by interfacial forces during drying might have given rise to filaments in the final image. However, their oval shape, their size (often decreased by osmosis, drying and electron bombardment), their similarity and incidence with the better characterized images from Fig. 6 and 8 to 10, as well as with unhemolyzed erythrocytes as in Fig. 1, the presence of filaments at the level of the upper portion of their seemingly collapsed envelopes and especially their marked similarity to known stromatolytic forms, all make their identification with stro-

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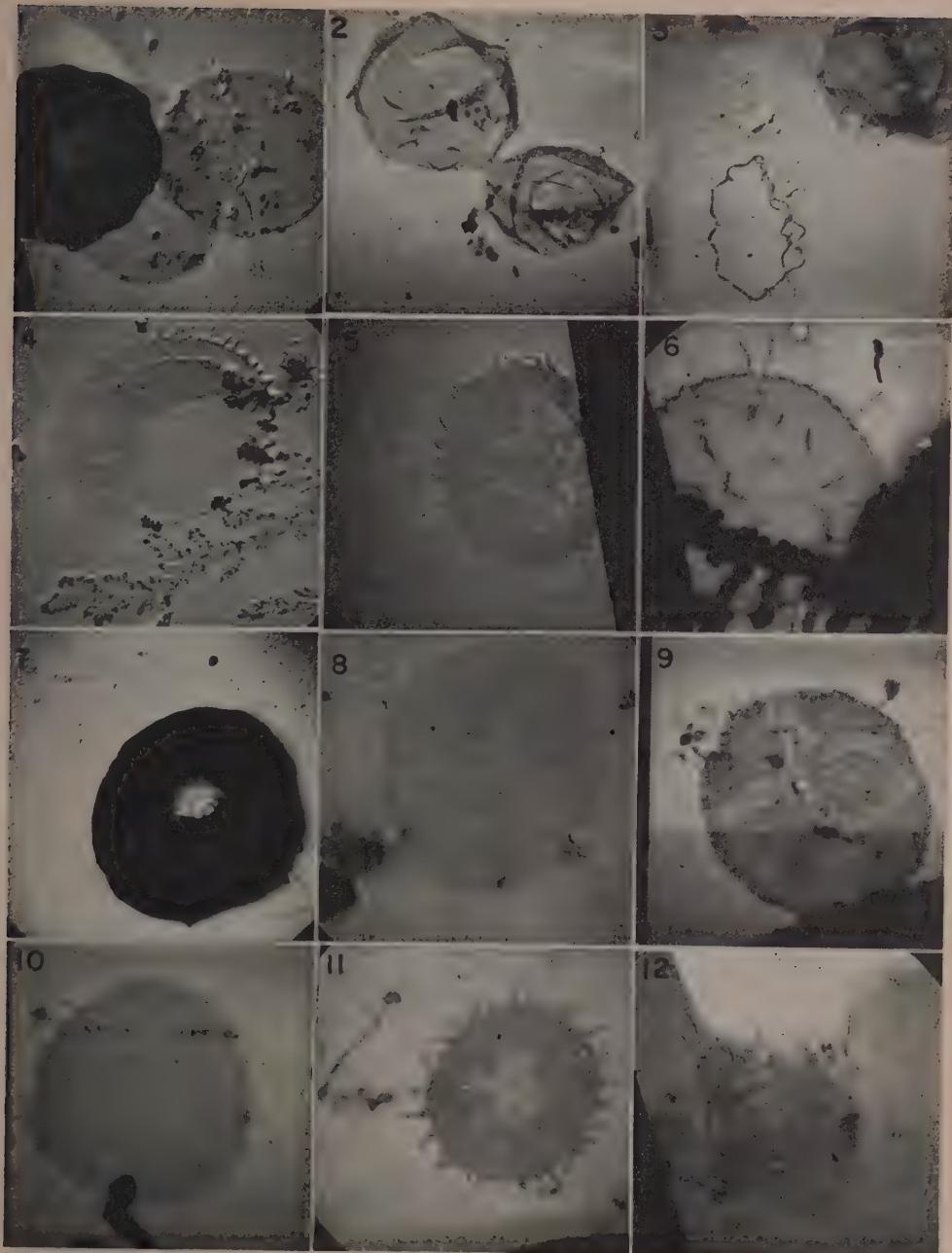


Fig. 1 to 5 and 7 and 12 shadow-cast with chromium at angle of about 12°. Magnification  $\times 4,000$ .

matolyzing erythrocytes highly probable. This contention receives added support from the formation of similar filamentous processes in

a large number of materials also rich in phospholipids (1,10).

Unhemolyzed erythrocytes are found in

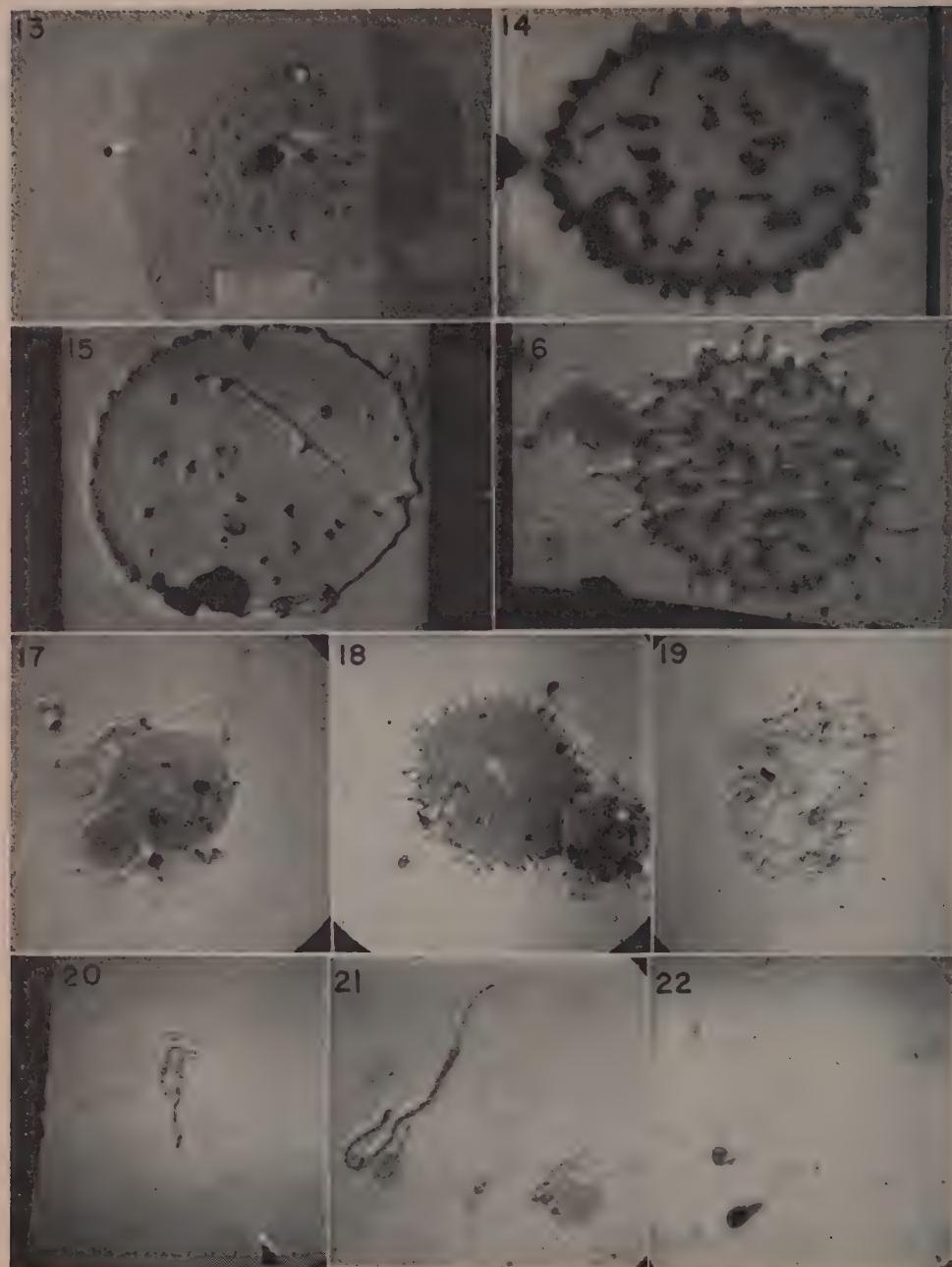


Fig. 13, and 15 to 21 shadow-cast with chromium at angle of about  $12^\circ$ . Magnification: Fig. 13,  $\times 6,000$ ; Fig. 14,  $\times 4,800$ ; Fig. 15,  $\times 5,600$ ; Fig. 16,  $\times 4,600$ ; Fig. 17 to 22,  $\times 4,000$ .

Fig. 1 and 7. Hemolyzed erythrocytes with no apparent change in configuration are il-

lustrated in Fig. 1. No processes are found in the erythrocytes from Fig. 2 except folds

in the erythrocyte envelope. According to de Robertis *et al.*(12), this envelope appears to correspond to the plasma membrane plus some external layers of cytoplasm. On the other hand, folds were observed in fixed chick embryo erythrocytes(11), but no folds have been seen with the electron microscope in chick embryo erythrocytes which were not subjected to chemical fixation. Probably the preparation of the specimens which was unlike that of the present case accounts for the difference observed. Folds are seen in torn erythrocytes (Fig. 3) and they can be seen also in erythrocytes showing short, relatively broad processes (Fig. 4).

The density of the periphery and of the processes themselves which arise from the periphery of the erythrocytes (Fig. 6 and 14) may be explained by persistence of hemoglobin at these levels. This is supported by the finding of partially hemolyzed (Fig. 9) and unhemolyzed (Fig. 1 and 7) erythrocytes in the same preparation. Unhemolyzed, non-nucleated erythrocytes often show a less dense center in electron micrographs which indicates less hemoglobin in this area (Fig. 1 and 7). This may support the contention that hemoglobin tends to persist at the cell periphery even in partially hemolyzed erythrocytes.

Seifriz(7) in his work on living human and amphibian erythrocytes remarks on how easily the cytoplasm flows into the processes formed on the cell surface. This fact apparently has some bearing upon the explanation for the images in Fig. 14, 16 and others. In these figures while much of the corresponding image is flattened as the envelope collapses under hemolysis, some hemoglobin has apparently been left at the level of the processes which consequently do not flatten to the same extent. The density of the processes in the unshadowed images (Fig. 6 and 14) and the raised appearance of these processes in shadowed fields, depicted in Fig. 4, 5 and 16, also support the same argument.

In Fig. 15 there are rounded, rectangular areas of high contrast which are very likely

images of sodium chloride crystals. Sodium chloride residues are seen also in several other fields, for example in Fig. 4, 17 and 18. Since the shadows cast by the processes are in general of a rather constant length, it is likely that they lie mostly in the object plane. The image from Fig. 13 shows dense areas of irregular contour and marked density, which throw no shadows and are likely the manifestation of dense areas within the envelope. The large particle which throws a shadow is a crystal.

*Discussion.* Many workers have reported appearances identical or similar to the crenated aspect shown by a number of the erythrocytes presented here. Seifriz(7) obtained evidence which supported the suggestion of Brinkman and Dam(13) that crenation of erythrocytes was not an osmotic phenomenon. Bechhold(4), Salén(5), and Rockwood(6) observed these stromatolytic forms in unhemolyzed erythrocytes from preparations where hemolysis was occurring. Kite(2), Oliver(3), Takeuchi(8) and Auer (9) did likewise in unhemolyzed erythrocytes which were standing under various other conditions. In a few cases these forms were found attached to other erythrocytes(8,9). Seifriz(7), in a study of living human and amphibian erythrocytes pointed out that changes in the physical properties of the erythrocyte envelope resulted in changes in the cell surface configuration. These changes could be provoked by microsurgical manipulation or could appear spontaneously. They consisted of pseudopod-like processes, globular extrusions and conical processes, all at the erythrocyte envelope, and papillae or filaments at the cytoplasm only. These papillae closely resemble many of the filaments depicted here, especially in the rather frequent terminal swelling (Fig. 1, 4, 5, 11 and 12). In some instances, the papillae observed by Seifriz left vacuolar depressions in the cell below the envelope, which are apparently identical with the depressions seen here in Fig. 5 and 11. A pseudopod-like process appears to have occurred in Fig. 13, while

12. de Robertis, E. D. P., Nowinski, W. W., and Saez, F. A., *General Cytology*, Philadelphia, W. B. Saunders Co., 1948, 122.

13. Brinkman, R., and Dam, E. van, *Biochem. Z.*, 1920, v108, 52.

globular and conical processes may be seen in Fig. 14 and 15, respectively. With the optical, dark field microscope, Furchgott(10) followed the process of disintegration of hemolyzed, human, beef, and calf erythrocytes. As the initial stage under the action of lyotropic salts, ghosts showed small buds at the cell periphery. Then, these buds progressed away from the ghost periphery, thus constituting filaments which in a further stage separated from the ghost either intact or broken into smaller threads. Finally, these smaller filaments disintegrated into small particles unresolvable in the ordinary, bright field microscope and whose diameter was estimated as about 100 millimicrons was the dark field microscope. It was technically impossible to follow the whole process of stromatolysis in the electron microscope, but isolated appearances descriptive of all the stages reported by Furchgott are represented and depicted in this report. Furchgott observed stromatolysis under certain conditions even by the action of normal saline solution and of distilled water.

If Fig. 14, 16 and 12 are examined consecutively, the order for the progress of filament formation is noticed, which follows Furchgott's observations(10) that the first production of globular processes subsequently narrows until definite filaments are formed. To a less extent, this is true of Seifriz's findings(7) too, since he stated that while changes at the erythrocyte envelope progressed from pseudopod-like processes, to globular, to conical extrusions, filaments were cytoplasmic processes protruding through the envelope. On the other hand, observing Fig. 7 to 12, a quite gradual development of the progressive stages of filament formation is noticed while globular or conical processes are apparently missing. That this developmental order may actually occur seems to be supported by the appearance from Fig. 4 as well as by Seifriz's remarks(7) on the cytoplasmic origin of filaments in contrast to the origin of the globular and conical processes he observed. However, it should be pointed out that both definite filaments and blunt and conical processes may be seen in the same erythrocyte (Fig. 5

and 16).

The identification of the appearances in these micrographs with the different stages of stromatolysis is supported further by the very close morphologic similarity between the images in Fig. 17 to 22 (the bundles of threads in Fig. 19, the isolated filaments in Fig. 20 to 22 and the apparent cellular debris in Fig. 17 to 22) and the late stages of stromatolysis as reported by Furchgott(10). Furchgott noticed the resemblance to the so-called myelin forms of many of his stromatolytic structures. The former arise from the myelin sheath of nerve fibers as well as from other biological structures and even from simple chemical compounds of phospholipid nature. In this regard he stressed the high phospholipid content of erythrocyte ghosts. From shadow-casting considerations, only those erythrocytes which are relatively unchanged by the handling (Fig. 1 and 7) have any residual thickness. All of the others have flattened to the surface of the supporting film in such a way as to throw very little shadow except at the periphery. No changes were observed in the specimens during electron bombardment, and if specimen changes did occur they must have been due to drying alone. Some of these effects have been discussed earlier(11).

Although it is not possible to follow erythrocyte stromatolysis directly with the electron microscope, this instrument does offer increased resolution over the light microscope, and with the aid of shadow-casting an additional advantage in contrast, especially over the cell surface. Increased detail of the stromalytic processes is then to be expected. The correlation of light and electron microscopic information is very valuable when interpretations are being made in such a study.

*Summary.* Widely different degrees of stromatolysis were observed electron microscopically in erythrocytes which occurred in preparations of *Treponema carateum* taken from tissue fluid of skin lesions. A comparison of electron images with light microscopic images of cells under controlled conditions of stromatolysis showed striking similarities. Isolated appearances in the electron micro-

graphs similar to all the stages of disintegration of hemolyzed erythrocytes reported by Furchtgott from light microscopy are repre-

sented and observed.

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## Action of Vitamin B<sub>12</sub> in Counteracting Glycine Toxicity in the Chick.\* (18125)

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Cary *et al.*(1) and Rubin and Bird(2), working with rats and chicks, respectively, have observed growth-inhibitory effects from high levels of protein in diets now known to be low in vitamin B<sub>12</sub>. McGinnis *et al.*(3) reported that the nonprotein nitrogen content of the blood in chicks fed diets high in protein and deficient in the "animal protein factor" was higher than in chicks fed the same diets supplemented with this factor. Zucker and Zucker(4) observed a similar condition in rats. Since that time, vitamin B<sub>12</sub> has been shown by Ott *et al.*(5), Lillie *et al.*(6), and others, to be an important part of the animal protein factor. Recently, Charkey *et al.*(7) have demonstrated that the levels of nonprotein nitrogen and amino acids in the blood were higher in vitamin B<sub>12</sub>-

deficient chicks than in chicks fed vitamin B<sub>12</sub> (Merck & Co., APF Supplement No. 3). These workers concluded that vitamin B<sub>12</sub> appears to function in metabolism by enhancing the utilization of circulating amino acids for building fixed tissues. These observations are in agreement with those noted in this laboratory (unpublished data).

In view of these findings, the present study was conducted to determine the effect of feeding different levels of glycine to chicks receiving various amounts of vitamin B<sub>12</sub>. Evidence is presented to indicate that vitamin B<sub>12</sub> is concerned in the metabolism of glycine in the chick.

*Experimental.* New Hampshire chicks of mixed sexes, obtained from dams kept on raised wire platforms and fed a ration low in vitamin B<sub>12</sub>, were used in these experiments. The chicks were maintained in electrically heated batteries, and feed and water were supplied *ad libitum*. All glycine supplements were made at the expense of cerelose (glucose). Merck & Co. APF Supplement No. 3 was used as the source of vitamin B<sub>12</sub>.

In Exp. 1, day-old chicks first were fed basal diet 122 (developed in this laboratory, Table I) during an 18-day preliminary period to further deplete them of vitamin B<sub>12</sub>. This diet was deficient in vitamin B<sub>12</sub> and contained 35% protein. At the end of the depletion period, six comparable groups of 15 chicks each were selected on the basis of body weight. Groups 1, 3, and 5 were fed the basal diet plus 0, 3, and 30 µg of vitamin B<sub>12</sub> per kilo, respectively. The other 3 groups were fed these same diets except that 1%

\* Scientific paper No. A287. Contribution No. 2233 of the Maryland Agricultural Experiment Station (Department of Poultry Husbandry). This work was supported in part by a grant from the Research Grants Division of the National Institutes of Health, United States Public Health Service.

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2. Rubin, M., and Bird, H. R., *J. Nutrition*, 1947, v34, 233.

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6. Lillie, R. J., Denton, C. A., and Bird, H. R., *J. Biol. Chem.*, 1948, v176, 1477.

7. Charkey, L. W., Wilgus, H. S., Patton, A. R., and Gassner, F. X., *PROC. SOC. EXP. BIOL. AND MED.*, 1950, v73, 21.

VITAMIN B<sub>12</sub> IN COUNTERACTING GLYCINE TOXICITY

TABLE I. Composition of Basal Diet 122.

	%
Cerelose	51.67
Alpha soybean protein	38.00
Soybean oil	3.00
Cod liver oil (3000 A; 400 D)	.50
DL-methionine	.60
L-leucine	.20
Mineral mixture 1 M*	6.00
Iodinated casein (Protamone)	.03
mg/100 g	
Thiamin HCl	1.00
Riboflavin	1.00
Calcium pantothenate	2.00
Pyridoxine HCl	.60
Niacin	5.00
Folic acid	.30
p-Aminobenzoic acid	.20
Menadione	.50
Biotin	.02
Choline chloride	200.00
i-Inositol	100.00
alpha-tocopherol acetate	.50

1200 I.U. vit. A, and 0.5 mg of alpha-tocopherol acetate were administered by dropper weekly.

\* Described by Briggs(8).

glycine was also added. A 3-week experimental period was employed.

In Exp. 2, the protein level of the basal diet was lowered to 21% by reducing the level of alpha soybean protein from 38 to 23%. Additional cerelose was used in compensation. A preliminary vitamin B<sub>12</sub>-depletion period was omitted in this trial since the parent stock had been maintained on the vitamin B<sub>12</sub>-deficient ration for a longer period of time. Six groups, each containing twelve day-old chicks, were fed the modified basal ration plus different supplements. Combinations of 0, 3, and 30 µg of vitamin B<sub>12</sub> per kilogram of diet, with and without 4% added glycine, were tested. This experiment was discontinued at the end of two weeks, since the addition of 4% glycine to the vitamin B<sub>12</sub>-low basal diet resulted in 75% mortality. Nevertheless, the inhibitory action of this level of glycine on vitamin B<sub>12</sub>-deficient chicks appears clearly evident even in this brief test period.

**Results and discussion.** The result obtained in Exp. 1 are given in Table II. It is evident that the growth of vitamin B<sub>12</sub>-deficient chicks was depressed by the addi-

tion of 1% glycine to the diet when no vitamin B<sub>12</sub> was supplied. This growth-inhibitory action of glycine was counteracted by the addition of either 3 or 30 micrograms of vitamin B<sub>12</sub> per kilogram of diet. The slight difference in the average gains of the two groups of chicks (Groups 5 and 6) fed diets containing 30 micrograms of vitamin B<sub>12</sub>, with and without added glycine, is not statistically significant. A previous study (unpublished) supports this conclusion. In that test, chicks fed basal diet 122, supplemented with 1% added glycine and 30 micrograms of vitamin B<sub>12</sub> per kilo, grew somewhat more

TABLE II. Effect of 1% Added Glycine in Combination with Various Amounts of Vitamin B<sub>12</sub> on Chick Growth. (Experiment I).

Group No.	Supplement to basal diet 122	No. of surviving chicks†	Avg gain during 3 wk exp. period (g)
1	None	14	146
2	1% glycine	13	99*
3	3 γ B <sub>12</sub> /kg	14	227
4	3 γ " + 1% glycine	14	209
5	30 γ B <sub>12</sub> /kg	15	336
6	30 γ " + 1% glycine	14	309

\* The difference between this value and that of group 1 is statistically significant to the 5% level.

† Each group contained 15 chicks at the start.

TABLE III. Effect on Chick Growth and Mortality Obtained from the Addition of 4% Glycine in Combination with Different Amounts of Vit. B<sub>12</sub>. (Experiment II).

Group No.	Supplement to modified basal diet	No. of surviving chicks*	Avg gain of surviving chicks during 2 wk exp. period (g)
1	None	11	42
2	4% glycine	3	36
3	3 γ B <sub>12</sub> /kg	12	65
4	3 γ " + 4% glycine	11	75
5	30 γ B <sub>12</sub> /kg	12	73
6	30 γ " + 4% glycine	12	71

\* Each group contained 12 chicks at the start.

rapidly than those fed the same diet without additional glycine.

The results of Exp. 2 are presented in Table III. The effect of 4% added glycine in the vitamin B<sub>12</sub>-deficient diet was striking. Only 3 of the 12 original chicks fed this diet (Group 2) survived the 2-week period. However, the addition of as little as 3 µg of vitamin B<sub>12</sub> per kilo of diet (Group 4) prevented the excessive mortality and completely counteracted the growth-inhibitory action of the added glycine, confirming the results obtained in Exp. 1. Since Merck's APF supplement No. 3 was used as the source of vitamin B<sub>12</sub>, the possibility exists that the effects observed may have been the result of something other than vitamin B<sub>12</sub>. This possibility, however, is not considered to be very great.

The inhibitory action of the 4% added glycine in the vitamin B<sub>12</sub>-deficient diet containing 21% protein (Exp. 2) cannot be attributed to its effect on the protein level of the diet, since the addition of 1% glycine to the 35% protein diet (Exp. 1) did not cause excessive mortality. Therefore, the retarded growth and extreme mortality which was observed in vitamin B<sub>12</sub>-deficient chicks fed the high level of glycine may be assumed to have resulted from a specific glycine imbalance rather than from a change in the protein level of the diet. Since the addition of vitamin B<sub>12</sub> to all diets containing 1% or 4% added glycine counteracted the inhibitory action of this amino acid on chick growth, it appears that vitamin B<sub>12</sub> functions in some manner in the metabolism of glycine.<sup>†</sup>

This finding is comparable with those of Groschke and Briggs(9) and Anderson *et al.* (10) of this laboratory, who found that niacin and pyridoxine, respectively, are also concerned in the metabolism of glycine. Groschke and Briggs(9) demonstrated that

<sup>†</sup> At the conclusion of this investigation, it was learned that Machlin *et al.* at the Poultry Section, B. A. I. Beltsville, Md., had obtained similar results.

9. Groschke, A. C., and Briggs, G. M., *J. Biol. Chem.*, 1946, v165, 739.

10. Anderson, J. O., Combs, G. F., and Briggs, G. M., *Poultry Sci.*, 1949, v28, 755.

glycine was highly "pellagragenic" when fed to chicks receiving a niacin-low diet. However, they found that as much as 6% glycine could be included in the chick diet with no adverse effects when an adequate amount of nicotinic acid was supplied. Anderson *et al.* (10) similarly showed that the addition of 4% glycine to a pyridoxine-low diet exerted a growth-depressing action in the chick. This was overcome by the addition of pyridoxine.

The growth-depressing effect of large amounts of glycine fed to rats and its counteraction by certain vitamins has been reported by other workers. Dinning *et al.*(11) and Martel *et al.*(12) observed that rats fed diets containing 10% glycine grew at a subnormal rate. A marked improvement in growth rate was noted upon addition of folic acid. Pagé and Gingras(13) demonstrated that large intakes of glycine inhibited growth of rats fed a pyridoxine-deficient diet, but that the addition of pyridoxine to the diet overcame this inhibition. Furthermore, Martin(14) reported that glycine was more toxic when fed at high levels to riboflavin-deficient rats than when fed to rats maintained on a diet adequate in riboflavin. The results of the present investigation together with those of the workers referred to above indicate that vitamin B<sub>12</sub>, nicotinic acid, pyridoxine, folic acid, and riboflavin are required in the metabolism of glycine. This appears to be particularly true when unusually large amounts of glycine are fed.

**Summary.** Vitamin B<sub>12</sub>-deficient chicks were fed vitamin B<sub>12</sub>-deficient basal diets supplemented with 0, 1 and 4% glycine in combination with 0, 3, and 30 µg of vitamin B<sub>12</sub> (supplied by Merck's APF Supplement No. 3) per kilogram. The growth of the chicks that received either 1 or 4% added glycine in the vitamin B<sub>12</sub>-deficient basal diets

11. Dinning, J. S., Keith, C. K., Day, P. L., and Totter, J. R., *Proc. Soc. EXP. BIOL. AND MED.*, 1949, v72, 262.

12. Martel, F., Pagé, E., and Gingras, R., *Rev. Canadienne de Biologie*, 1947, v6, 802.

13. Pagé, E., and Gingras, R., *Trans. Roy. Soc. Can.*, 1946, v40, 119.

14. Martin, G. J., *PROC. SOC. EXP. BIOL. AND MED.*, 1946, v63, 528.

was depressed. Those chicks which received 4% added glycine without vitamin B<sub>12</sub> suffered excessive mortality. The addition of as little as 3 micrograms of vitamin B<sub>12</sub> per kilo of diet overcame the inhibitory action of both levels of glycine. These results indicate that vitamin B<sub>12</sub> functions in the metabolism of glycine.

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Co., Inc., Rahway, N. J., for the vitamin B<sub>12</sub> concentrate and other crystalline vitamins; Lederle Laboratories, Pearl River, N. Y., for folic acid; Allied Mills, Inc., Portsmouth, Va., for soybean oil; Abbott Laboratories, North Chicago, Ill., for Haliver oil; U S. Industrial Chemicals, Inc., New York City, N. Y., for DL-methionine; and Cerophyll Laboratories, Inc., Kansas City, Mo., for Protamone.

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### Inhibition of Calcification *in vitro* by Surface Active Compounds.\* (18126)

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The observation that some types of pathological lipid deposition are often followed by mineralization suggests that lipid substances may enhance mineral salt deposition. An *in vitro* study of the role of lipids in the calcification of hypertrophic epiphyseal cartilage of the rachitic rat may further our understanding not only of normal calcification, but may also provide a clue to the nature of abnormal mineralization. This method, which enables one to observe the selective formation of new bone salt in rachitic epiphyseal cartilage, depends on the functioning of the complete system essential for calcification (1-3, 6, 11, 12). This technic has provided, in previous experiments, evidence that lipids are

related to calcification. For example, bone formation is inhibited in the rachitic tibia if it has been previously extracted with alcohol, acetone, or chloroform (2). Moreover, the calcifying mechanism is injured by phloridzin and iodoacetic acid; two compounds which are inhibitors not only of phosphorylative glycogenolysis, but also of fat absorption (3,4). That these inhibitions can be overcome with excess inorganic or organic phosphates does not preclude the possibility that a system handling lipids is directly involved in the local deposition of bone salts (5,6). Levine and Follis (7,8) have demonstrated the presence of a lecithinase in cartilage. They postulated that it is part of an independent system for elevating the local concentration of phosphate ions. Another line of investi-

\* This investigation was made possible by a grant from the Wendell Willkie Memorial Fund. A preliminary report appeared in *Am. Chem. Soc. Abst.*, 117th Meeting, 1950.

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TABLE I. Inhibition of calcification *in vitro* by Surface Active Compounds.\*

Surface active agent	Cone. of agent	Degree of calcification*	Degree of control calcification*
Sodium desoxycholate	$1.9 \times 10^{-4}$ M	1(++++)	1(++++)
" "	$1.9 \times$ "	2(++++)	2(++++)
" "	$4.8 \times$ "	0	1(++++)
" "	$4.8 \times$ "	0	2(++++)
Sodium cholate	$0.9 \times 10^{-3}$ M	2(++++)	2(++++)
" "	$1.9 \times$ "	(+++)	2(++++)
" "	$1.9 \times$ "	(+++)	1(++++)
" "	$2.8 \times$ "	0	1(++++)
Duponal C	0.3 x "	1(++++)	2(++++)
" "	$0.7 \times$ "	1(++++)	2(++++)
" "	$0.7 \times$ "	1(++++)	1(++++)
" "	$1.4 \times$ "	(++)	1(++++)
" "	$2.8 \times$ "	0	1(++++)
Tween-20	0.04%	2(++++)	2(++++)
" "	0.08%	(+++)	2(++++)
" "	0.08%	(+++)	1(++++)
" "	0.20%	0	1(++++)
	0.40%	0	1(++++)

\* Specimens incubated in the calcifying solution 18-24 hr. Calcifying solution contains: .7 eq/L NaCl, .05 eq/L KCl, .22 eq/L NaHCO<sub>3</sub>, Ca x P product = 10 mg Ca and 3 mg P = 30. The degree of calcification is indicated as follows: 0 = no calcification; (+) trace; (++) broken thin line; (++) almost complete thin line across the provisional zone; (++) complete thin line across the provisional zone; 1(++++) heavy line across the provisional zone including the primary tongues of cartilage; 2(++++) heavy line across the provisional zone including the primary and secondary tongues of cartilage; 3(++++) practically complete calcification of the metaphysis.

gation which indicates that lipids are related to calcification, is the work of Levander(9). He demonstrated the presence of an alcohol soluble factor in embryonic rabbit bone which initiated osteogenesis in rabbit muscle. His results have been questioned by Pfeifer(10).

In the present experiments, the effect of a variety of surface active agents on calcification *in vitro* was studied in an attempt to alter the lipid equilibria of calcifying cartilage. Wistar strain albino rats, 22-24 days of age, were placed on a modified Steenbock-Block diet for 21 days(11). Thin slices of epiphyseal cartilage were cut from the tibia by hand and incubated at 37°C without shaking in sealed flasks in the calcifying solution containing the concentrations of surface active agents described in Table I. The flasks were stoppered so as to prevent gas exchange with the atmosphere. During the 18-24 hour period of incubation, the pH was maintained at 7.35-7.4. The sections were then washed with distilled water and stained with 2% silver nitrate to reveal the newly formed bone salt deposits. Variations in the response of the calcifying mechanism of individual rats were controlled by incubating untreated sec-

tions from each animal used in a particular experiment. It was found that the surface active agents used, blocked the formation of bone. As can be seen in Table I, sodium desoxycholate was effective at a concentration of  $4.8 \times 10^{-4}$  M and another bile salt, sodium cholate was effective at a concentration of  $1.9 \times 10^{-3}$  M. Of the 2 synthetic detergents tried, the anionic Duponal C inhibited in a concentration of  $1.4 \times 10^{-3}$  M and the nonionic Tween-20 inhibited at a concentration of 0.08%. By varying the standard technic, a difference in the effect of the above surface active agents was noted, particularly with Tween-20. This modified procedure consisted of shaking rachitic sections with various higher concentrations of these compounds for 7 minutes in an unstoppered flask at 25°C, washing with distilled water, and then incubating the tissue in the calcifying solution in the manner described above. As shown in Table II, with less than 40 times the minimum concentration of the respective compound required to inhibit calcification with the continuous treatment in Table I, various degrees of inhibition were found with sodium desoxycholate, sodium cholate, and Duponal

TABLE II. Inhibition of Calcification *in vitro* by Preliminary Shaking with Surface Active Compounds.

Surface active agent	Cone. of agent	Degree of calcification*	Degree of control calcification*
Sodium desoxycholate	1.2 × 10 <sup>-2</sup> M	0, 0	1(++++)
	1.2 × " "	0, 0, 0	
Sodium cholate	5.8 × "	1(++++)	2(++++)
	5.8 × "	1(++++)	
Duponal C	5.8 × "	(+), (+++)	1(++++)
	5.3 × "	(+), (+++)	
Tween-20	5.3 × "	0, 0, 0	2(++++)
	5% —	1(++++)	
	20%	2(++++)	2(++++)

\* The incubation period, composition of the calcifying solution, and the coding methods are the same as used previously in Table I. The pH of the preliminary shaking solutions varied between 7.2 and 8.

C. However, with Tween-20 it was possible to increase the concentration 250-fold without observing any trace of inhibition. Previous work has shown that preliminary shaking with high concentrations of sodium chloride for 2 hours will inhibit calcification(12). The variation in sodium concentration in the above compounds, however, can not be correlated to the degree of inhibition.

It is possible that one is dealing with more than one type of inhibition in the above experiments. Not only may bile salts and anionic detergents interact with lipids, but they may also modify proteins and activate as well as inactivate various enzymes(13). In dealing with tissue slices containing a complex biological system, it is difficult to draw conclusions based on detergent action in simple solutions. In the standard *in vitro* procedure described above, the low concentrations in which the bile salts and Duponal C are effective are probably below the critical value required for the denaturation of proteins. Anson(14) treated soluble proteins with from 1/5 to more than equal their weight of detergent to produce denaturation. Furthermore, Hotchkiss(15) has pointed out, "The concentrations of detergent capable of denaturing most ordinary soluble proteins are in a higher range than those necessary for killing bacteria." Therefore, the question

as to whether cellular damage occurred which was responsible for the observed inhibitions with low concentrations of these agents must be seriously considered. The properties of the nonionic Tween-20, which inhibited only when present during the actual period of bone salt deposition, strongly suggests that possible cellular damage is not a critical factor in causing inhibitions with low concentrations of detergents. Tween-20, as a nonionic type of detergent does not inhibit bacterial growth(15) and hence would not be expected to injure any cells which may be involved in calcification. Moreover, since proteins and nonionic detergents have not been demonstrated to interact(16), the cause of inhibition by Tween-20 may be related to some alteration of the lipid equilibria essential for calcification. It is possible that the nature of the inhibition by low concentrations of the other agents is similar to that which occurs with Tween-20.

In the modified procedure, the concentrations of bile salts and Duponal C are sufficiently high so that the possibility that intracellular and extracellular lipid and protein components of the calcifying system have been modified exists(13,16). Metabolic studies were not undertaken to determine whether cells were in fact injured. However, the lack of inhibition by Tween-20 under these modified conditions, further indicates the presence of a more sensitive type of inhibition to the

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calcifying system when lower concentrations of surface active compounds are present during the actual process of mineralization. The existence of an essential lipid factor secreted by the cells as calcification proceeds would help explain the observed effect of the above surface active agents on the calcifying mechanism.

*Summary.* Calcification *in vitro* of the hypertrophic epiphyseal cartilage was found

to be inhibited by  $4.8 \times 10^{-4}$  M sodium desoxycholate,  $1.9 \times 10^{-3}$  M sodium cholate,  $1.4 \times 10^{-3}$  M Duponal C, and .08% Tween-20. By preliminary treatment of rachitic sections with higher concentrations of these surface active agents for a short time interval, it was possible to demonstrate inhibition only with the bile salts and Duponal C.

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### The Effect of X Radiation on Antibody Formation.\* (18127)

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Hektoen(1) conclusively demonstrated that total-body exposure of experimental animals to ionizing radiation suppressed the usual antibody response to antigens injected shortly before or shortly after irradiation. Hektoen's observations led him to ascribe this suppression to the destructive effect of irradiation on lymphatic tissue and bone marrow. These findings have been corroborated by a number of workers and have recently been reviewed by Taliaferro(2). Craddock and Lawrence(3) reported that total-body exposure of rabbits to 250 r X radiation effectively suppressed the formation of antibodies to antigens (typhoid vaccine and washed red cells from sheep) administered 8 hours after irradiation.

This communication relates a study on the capacity of the rabbit to form antibodies after the whole body, except for the spleen or appendix, has been exposed to 800 r X radia-

tion. A preliminary report on this work was described elsewhere(4).

*Materials and methods.* Young adult rabbits (Swift's snuffle-free), weighing approximately 2 kg were used in this study. The rabbits were divided into groups and the experiments carried out as indicated in Tables I and II.

The rabbits of all groups, including the control animals, were anesthetized with Nembutal (38 mg per kg total-body weight) administered intravenously in the first experiments; later only those were given anesthetic which were subjected to irradiation, surgical exteriorization of the spleen, or both. Under anaesthesia, the spleen of each animal of Groups 3, 7, 8, and 9 was withdrawn from the abdominal cavity through a left upper quadrant incision. In the animals in Group 3, which were not irradiated, the spleen was exteriorized, wrapped in moist gauze, and left for one hour (the approximate time required to deliver the radiation to the exposure groups). In Group 7 the spleens were placed in lead shields during irradiation. The lead shields consist of two parts that fit together by means of overlapping flanges with a baffle type slit 3/16-inch wide and measuring 3 1/4 inches along the long axis. The wall of the

\* Supported in part by a grant from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council, and (in part) by a grant from the National Cancer Institute, U. S. Public Health Service.

1. Hektoen, Ludvig, *J. Infect. Dis.*, 1915, v17, 415.  
2. Taliaferro, W. H., and Taliaferro, Lucy G., The Effect of X-rays on Immunity, Metallurgy Lab., University of Chicago, AECU-240, CH-3891, June 1946.

3. Craddock, C. G., and Lawrence, J. S., *J. Immunol.*, 1948, v60, 241.

4. Jacobson, L. O., Robson, M. J., Marks, E. K., and Goldman, M. O., *J. Lab. Clin. Med.*, 1949, v34, 1612.

## X RADIATION ON ANTIBODY FORMATION

TABLE I. Effect of Lead Shielding of the Surgically Mobilized Spleen on Antibody Response.

Group	Rabbit No.	Preparation	Antigen	Hemolysis titer at intervals after immunization						
				Control	7 days	10 days	14 days	21 days	28 days	35 days
1	1	None	1 cc 2% sheep cells	0	40	5120	5120	640	—	—
	2		I.V.	10	40	1280	160	80	20	
	3			0	0	1280	1280	320	320	
	4			0	640	640	640	320	80	80
	5			10	2560	5120	5120	1280	640	
	6			10	640	640	640	320	320	
	7			40	1280	2560	1280	320	640	
2	1	None	None	—	0	0	20	0	0	
	2			—	0	10	0	0	0	10
	3			—	0	80	20	10	20	10
	4			—	0	20	20	20	10	0
	5			—	0	0	0	10	0	0
3	1	Spleen exterioriza-	1 cc 2% sheep cells	0	0	80	40	0	0	
	2	tion only	I.V.	0	1280	2560	5120	5120	2560	
	3			0	2560	5120	640	320	320	160
	4			0	1280	1280	2560	1280	160	
	5			10	1280	2560	320	—	—	
	6			0	1280	640	1280	1280	—	
	7			20	320	—	—	—	—	
4	1	Splenectomy only 24 hr prior to antigen injection	1 cc 2% sheep cells	0	40	5120	5120	320	—	—
	2		I.V.	0	2560	2560	5120	5120	2560	
	3			0	10	20	40	40	40	10
5	1	800 r total-body irradiation only	1 cc 2% sheep cells	0	0	0	0	0	—	—
	2		I.V.	0	0	0	0	20	40	
	3			0	0	0	0	80	—	
	4			0	10	* 10	40	40	40	80
	5			*	0	0	0	10	0	0
6	1	800 r total-body irradiation	None	20	0	—	—	—	—	—
	2			0	0	0	0	0	0	0
7	1	800 r total-body irradiation with spleen shielding	1 cc 2% sheep cells	0	0	320	—	—	—	—
	2		I.V.	0	0	40	80	5120	20	
	3			0	40	160	640	0	0	
	4			0	80	160	320	20	80	
	5			0	0	80	1280	5120	80	
	6			0	40	80	40	0	0	0
	7			0	0	160	160	20	20	20
	8			0	0	20	40	*	80	10
	9			0	640	320	320	160	160	
	10			0	0	10	*	160	160	
	11			10	10	20	—	—	—	
8	1	800 r total-body irradiation with spleen exteriorization but no shielding	1 cc 2% sheep cells	0	0	0	0	—	—	—
	2		I.V.	0	0	0	0	—	—	—
	3			10	10	0	—	—	—	—
	4			0	0	0	0	0	0	
	5			0	0	0	0	0	0	
	6			0	0	0	0	80	80	40
	7			0	0	0	10	10	10	10
	8			0	0	0	0	0	0	
	9			0	0	0	0	0	0	
	10			0	0	0	0	—	—	
	11			0	0	0	0	0	0	

TABLE I. (Continued).

Group	Rabbit No.	Preparation	Antigen	Hemolysis titer at intervals after immunization						
				Control	7 days	10 days	14 days	21 days	28 days	35 days
9	1	800 r total-body irradiation with spleen shielding, splenectomy 3 days after irradiation	1 cc 2% sheep cells I.V.	0	20	160	320	—	—	—
2	3	Same as above except splenectomy 4 days after irradiation	1 cc 2% sheep cells I.V.	0	40	40	20	40	20	
4	5	800 r total-body irradiation with spleen exteriorization but no shielding, splenectomy 3 days after irradiation	1 cc 2% sheep cells I.V.	10	0	0	0	—	—	—
6		Same as above except splenectomy 4 days after irradiation	1 cc 2% sheep cells I.V.	0	0	0	0	0	0	0
7		Spleen exteriorization, no radiation, splenectomy 3 days after exteriorization	1 cc 2% sheep cells I.V.	0	1280	5120	2560	1280	1280	
8		Same as above 4 days after exteriorization	1 cc 2% sheep cells I.V.	0	320	640	1280	640	320	
9		No preparation, but splenectomized same as above	1 cc 2% sheep cells I.V.	10	80	160	160	160	160	

— Death of animal.

\* Serum not obtained.

cylinder is  $\frac{1}{4}$ -inch thick. This arrangement prevents compression of the splenic pedicle and minimizes the penetration of X rays or scatter of X rays into the cylinder.

Twenty-four hours after operation and X irradiation, each animal in groups 1, 3, 4, 5, 7, 8, and 9 received 1 cc of a 2% suspension of washed sheep cells intravenously. Blood specimens were obtained from all rabbits before treatment ("Control" in Tables I and II) and on those which still survived at 7, 10, 14, 21, and 28 days after immunization. Blood was drawn for study at the 35-day interval in only a few animals.

After it was established, as described in the results below, that antibody production was not universally suppressed when the spleen was shielded during irradiation, an additional experiment was performed (Group

9). On the third and fourth days following irradiation and spleen shielding (2 and 3 days after immunization) splenectomies were done on 12 rabbits—2 from each of the irradiated groups (protected and not protected) on each day and one each from the control animals (normal and operation only). Streptomycin (75 mg/rabbit) was given to all animals (control included) on the day of irradiation and spleen exteriorization and was continued through the sixth day in a dose of 225 mg/day in three divided doses. None of the other groups of rabbits received an antibiotic. Splenectomy was performed on these animals using Nembutal anesthesia. The healed incision of the original surgery was reopened using clean surgical technic. The blood supply of the pedicle was tied off and the spleen removed. Any accessory spleens

## X RADIATION ON ANTIBODY FORMATION

TABLE II. Effect of Lead Shielding of the Surgically Mobilized Appendix on Antibody Response.

Group	Rabbit No.	Preparation	Antigen	Hemolysis titer at intervals after immunization						
				Control	7 days	10 days	14 days	21 days	28 days	35 days
10	1	None	1 cc 2% sheep cells	0	40	640	640	640	40	
	2		I.V.	0	1280	640	640	640	320	
	3			10	2560	5120	5120	5120	1280	320
	4			0	320	640	320	160	160	
	5			10	5120	1280	1280	1280	320	
11	1	None	None	0	0	0	0	0	—	—
	2			0	0	*	0	0	0	—
	3			0	0	0	0	0	0	0
12	1	Appendix exteriorization only	1 cc 2% sheep cells	10	2560	5120	5120	1280	640	
	2		I.V.	0	1280	5120	2560	640	320	160
	3			0	20	160	320	40	40	
	4			0	640	640	640	—	—	
	5			0	160	320	320	160	80	
	6			10	640	—	—	—	—	
	7			0	1280	1280	640	320	320	
	8			0	1280	640	320	160	160	
	9			0	640	320	320	320	320	
	10			0	2560	5120	5120	1280	630	
13	1	800 r total-body irradiation only	1 cc 2% sheep cells	0	0	0	0	0	0	0
	2		I.V.	0	0	0	0	0	—	—
	3			0	0	0	—	—	—	—
14	1	800 r total-body irradiation only	None	0	0	0	0	0	0	0
15	1	800 r total-body irradiation with appendix shielding	1 cc 2% sheep cells	0	0	0	0	640	—	—
	2		I.V.	0	0	40	320	80	80	
	3			0	40	320	320	320	160	80
	4			20	80	2560	5120	640	320	640
	5			0	0	20	640	640	80	
	6			0	0	160	1280	320	320	
	7			0	0	80	320	160	80	
	8			0	0	—	—	—	—	
	9			0	20	320	320	—	—	
	10			0	0	20	80	40	20	
	11			10	10	20	—	—	—	
	12			0	0	20	40	20	—	
	13			0	0	80	40	80	80	
	14			10	0	320	640	320	160	
	15			0	0	40	80	40	40	
	16			10	0	20	80	160	160	
	17			0	0	5120	5120	2560	320	
	18			0	40	640	640	640	320	
16	1	800 r total-body irradiation with appendix exteriorization but no shielding	1 cc 2% sheep cells	0	0	0	—	—	—	—
	2		I.V.	0	0	0	0	10	40	
	3			0	0	0	0	0	10	
	4			0	0	0	0	0	0	10
	5			0	0	0	0	0	0	0
	6			0	0	0	0	0	0	0
	7			0	0	0	0	0	0	0
	8			0	0	0	—	—	—	
	9			0	0	0	—	—	—	
	10			10	80	40	20	80	40	
	11			40	0	0	0	40	40	
17	1	800 r total-body irradiation with appendix shielding, appendectomy 5 days after irradiation	1 cc 2% sheep cells	0	40	5120	5120	5120	5120	
			I.V.							

TABLE II. (Continued).

Group	Rabbit No.	Preparation	Antigen	Hemolysis titer at intervals after immunization						
				Control	7 days	10 days	14 days	21 days	28 days	35 days
17	2	Appendix exteriorization, appendectomy 5 days later	1 cc 2% sheep cells I.V.	40	320	640	160	80	80	
	3	No preparation but	1 cc 2% sheep cells	0	160	160	160	320	160	
	4	appendectomy same as above.	I.V.	0	5120	5120	5120	2560	—	—

— Death of animal.

\* Serum not obtained.

that were found were also removed. Blood samples for titrations were taken just prior to surgery.

The hemolysin titers were determined by making serial dilutions of the rabbit sera (1:10, 1:20, 1:40, etc., to 1:5120) in 0.5 cc amounts, adding 0.5 cc of lyophil guinea pig complement and 0.5 cc of a 2% suspension of washed sheep red cells, incubating 1 hour at 37°C, and refrigerating overnight. The hemolysin titrations on each series (those animals treated and immunized on the same day) were done at the same time to eliminate differences in complement or sheep cell suspension. The titer of each particular serum was that dilution that gave complete macroscopic hemolysis.

*Experiments involving the appendix.* In the experiments involving lead shielding of the surgically exteriorized appendix (Table II, Groups 10-17) the same general technics were employed as were used in the experiments involving spleen shielding. A surgical incision was made in the right lower quadrant of the abdomen and the appendix was brought out and placed in a cylindrical lead container (1/4-inch thick throughout). This shield had an opening at one end for the proximal end of the appendix and a narrow baffletype slit with overlapping flanges to allow the blood supply of the appendix to remain intact during this procedure. The antigen was given 24 hours after irradiation.

On the fourth day following irradiation (third after immunization) appendectomy was done on 5 rabbits (Group 17). Two of these had lead shielding of the exteriorized appendix during irradiation, two had appendix

exteriorization during irradiation, but no shielding, and one had had its appendix exteriorized but received no radiation. These rabbits were anesthetized with Nembutal, and sterile surgical technic was employed. The incision of the original surgery was reopened. The blood supply to the appendix was tied off and a purse string suture was used to close the stump of the appendix. Only one animal which was irradiated in Group 17 (Table II, No. 1) survived the procedure. No antibiotics were used on these animals.

*Dosimetry.* The X rays were generated in a 250-kv machine operating at 15 ma. A 1.0-mm Cu filter and a 3.0-mm Bakelite filter were used. The half-value layer in copper of the filtered beam was 2.0 mm. The exposures were measured with a Victoreen condenser r-meter equipped with a 100-r chamber. Measurements were made in air at the position occupied by the center of the animal's body. The dose rate averaged 15.5 r per minute at 33 inches; a total dose of 800 r was delivered.

The amount of X radiation that the lead-protected appendix received has not been accurately measured. One-quarter inch of lead effectively eliminates appreciable penetration of X rays. Scatter through the slit opening of the appendix shield is probably minimal but some radiation enters the open end of the appendix shield.

*Histologic studies.* Groups of rabbits paralleling the spleen- and appendix-protected animals were set up in order to determine the effect of lead shielding of the appendix and immunization upon the cell population of these lead-shielded tissues. At least two ani-

mals from each group were sacrificed at intervals of 2, 7, 14, 21, 28, and 35 days after immunization. These sacrifice intervals were chosen since they represented the intervals at which blood was drawn for antibody titer studies. The 3-day interval was chosen since previous experience<sup>(5,6)</sup> had shown that at this interval destruction and atrophy of the hematopoietic tissues was generally at its maximum after this dose of X radiation. In this report, only the histologic findings at 3 days after irradiation are reported. In another experiment, to be reported<sup>(7)</sup>, rabbits exposed to 800 r or 1000 r total-body X radiation with lead protection of spleen or appendix were sacrificed for histologic study at intervals beginning at one day after irradiation through 35 days after irradiation. The histologic findings were identical with those observed in animals sacrificed in this present study of antibody formation. The tissues taken at autopsy for study were spleen, appendix, thymus, mesenteric lymph nodes, liver, kidney, adrenals, bone marrow (sternum and one femur), and intestine. These were fixed in Zenker-formol, embedded in 20% nitrocellulose, sectioned at 6 or 8  $\mu$ , and stained with hematoxylin-eosin-azure II.

*Results.* A dose of 800 r total-body X irradiation is followed by death of approximately one-half of the animals exposed to this dosage (LD<sub>50</sub>). The mortality of the irradiated animals which had surgery was not as high as in animals irradiated without surgery. Mortality was higher in our early experiments but as our surgical technic improved so did survival. No significance should be attached to these survival data because of this latter fact.

*Experiments involving lead-shielding of the spleen.* Animals given 800 r total-body X radiation without spleen shielding but which were immunized one day later (Groups 5 and 8, Table I) either developed no demonstrable

hemolysin titer, or titers of 1:80 or 1:40 were demonstrated on the twenty-first day, the twenty-eighth day, or thirty-fifth day, respectively, after immunization. In the normal animals (Group 1) and in animals that were subjected to surgical exteriorization of the spleen (Group 3) or splenectomy (Group 4) hemolysin titers as high as 1:5120 were produced by the tenth day; the titers had diminished by the twenty-eighth day after immunization. The hemolysin titers of individual rabbits given 800 r total-body X radiation exclusive of the surgically-exteriorized, lead-shielded spleen (Group 7) likewise reached values of 1:5120 but only by the twenty-first day. In general, the hemolysin titers in this group (Group 7) were lower and reached a maximum titer later than did normal controls (Group 1) or the additional control Groups 3 and 4. It is perhaps worth emphasizing that whereas the hemolysin titers of the control groups reached fairly high values by 7 days the titers of the spleen-shielded group (Group 7) as well as the appendix-shielded group (Group 15) had usually not risen by this interval. Animals in Group 9 (total-body X irradiation and spleen shielding) which were splenectomized three days after irradiation (2 days after antigen injection) or four days after irradiation (3 days after antigen injection) retained the capacity to produce hemolysin titers significantly higher than control irradiated nonshielded animals.

*Lead protection of the appendix.* Individual rabbits given 800 r total-body X radiation exclusive of the surgically-mobilized appendix and immunized twenty-four hours later produced antibodies reaching titers essentially comparable to the control groups; others, though producing titers higher than animals without lead shielding, were in some instances rather low (Table II). In general, the maximum titers in Group 15 were reached at 14 days. In rabbits given total-body X radiation including the appendix, the formation of antibodies was suppressed during the period of observation with the exception of an occasional animal which attained titers of 1:80 by 21, 28, or 35 days. Only one animal from Group 17, which had an appendectomy on

5. Jacobson, L. O., Marks, E. K., and Lorenz, E., *Radiology*, 1949, v52, 371.

6. Jacobson, L. O., Marks, E. K., Gaston, E., and Block, M. H., *J. Lab. Clin. Med.*, 1949, v34, 902.

7. Unpublished data.

the fifth day after irradiation (4 days after antigen injection) survived but this animal, which originally had 800 r total-body X irradiation and appendix shielding, attained a hemolysin titer of 1:5120 on the tenth day.

*Histologic studies.* Previous reports by Bloom *et al.*(8) and Jacobson *et al.*(5,6) have described the effects of a dose of 800 r total-body X radiation on the hematopoietic tissues of the rabbit. The lymph nodes throughout the body, the thymus, the patches of Peyer in the intestine, the appendix, and the spleen pass through a phase in which there is disintegration of the lymphocytes and virtually complete phagocytosis of the resultant particulate debris within the first 24 hours after irradiation. By 3 days the lymph nodes and spleen are practically devoid of lymphocytes, and the nodules appear as shrunken nests of reticular cells. The thymus is reduced to a sheath of epithelial cells in the cortex and the medulla is usually atrophic and devoid of lymphocytes. The bone marrow reaches a largely atrophic stage by 3 days after irradiation. Although regeneration of the bone marrow begins at about the 9th to 12th day and is orderly, normal cellularity is not attained before 14 to 21 days. The lymphatic tissue is reconstituted more slowly. Nodules rarely appear in the lymph nodes and spleen before the 12th day and usually not before the 14th to 21st day. Aggregates of lymphocytes begin to appear about the blood vessels and in the diffuse lymphatic tissue of the cortex and medulla of the lymph nodes about the 7th to 8th day after irradiation and increase slowly in number thereafter. In the experiment here reported the lymphatic tissues and bone marrow of all the irradiated groups were atrophic at 3 days with the exception of the spleen and the appendix of the groups that had either one or the other of these organs lead-protected during irradiation. In other words, whereas the lead-shielded spleen of irradiated animals remained essentially normal in size and cellularity, the marked cellular destruction and

reduced cellularity of the irradiated lymph nodes, thymus, intestinal lymphatic tissue, including the patches of Peyer and the appendix, and bone marrow observed at 3 days were indistinguishable from those observed in animals which received total-body exposure without lead-shielding of the spleen. Likewise, in animals which had lead-shielding of the appendix during irradiation, the appendix was qualitatively normal in size and its cellularity largely indistinguishable from the normal appendix of nonirradiated controls at 3 days; whereas, the hematopoietic tissue elsewhere was atrophic.

It is worthwhile emphasizing that no increase in plasma cells in the lead-shielded spleen or appendix was apparent at 3 days or at subsequent intervals. The irradiated lymphatic tissue of rabbits with lead-shielding of the spleen or appendix regenerated much more rapidly than the animals irradiated without spleen or appendix shielding(7). By 4 days active nodules were present in the lymph nodes of the irradiated animals with spleen or appendix protection. Recovery of the white pulp of the spleen was prominent by 4 days in animals with appendix protection; in animals with spleen protection, recovery of the appendicular lymphatic tissue was well underway by 4 days.

*Discussion.* In a previous communication(8) it was reported that the 28-day LD<sub>50</sub> for mice exposed to total-body X radiation exclusive of the surgically exteriorized, lead-shielded spleen is nearly twice as great (1025 r) as the LD<sub>50</sub> for mice exposed to total-body X radiation including the spleen (550 r). It has also been demonstrated (10,11) that lead protection of the surgically-exteriorized spleen of mice during the delivery of 600 r total-body X radiation obviated the development of anemia and significantly less-

9. Jacobson, L. O., Marks, E. K., Robson, M. J., Gaston, E., and Zirkle, R. E., *J. Lab. Clin. Med.*, 1949, v34, 1538.

10. Jacobson, L. O., Simmons, E. L., Bethard, W. F., Marks, E. K., and Robson, M. J., *PROC. SOC. EXP. BIOL. AND MED.*, 1950, v73, 455.

11. Jacobson, L. O., Simmons, E. L., Marks, E. K., Robson, M. J., Bethard, W. F., and Gaston, E. G., *J. Lab. Clin. Med.*, 1950, v35, 746.

sened the severity and duration of the leucopenia and thrombocytopenia that regularly follow total-body exposure at this level. In fact, no anemia of significance and only a transient leucopenia and thrombocytopenia occur after exposure of mice to 1025 r total-body X radiation if the surgically-exteriorized spleen is lead-shielded during delivery of the radiation. Regeneration of the bone marrow and lymph nodes of these mice is largely complete by eight days; whereas, no regeneration or a feeble attempt at regeneration characterizes animals exposed to this dose of irradiation without spleen shielding. It is apparent that the spleen, under these circumstances, assumes functions that are of signal importance in increasing survival following radiation exposure. The capacity of the spleens of these mice to compensate so quickly for the destruction of hematopoietic tissue elsewhere in the body as well as to hasten regeneration of the irradiated hematopoietic tissue may be the significant factor. On the other hand, the retention of the capacity to produce antibodies, as is demonstrated in this paper, may play a role also in increasing survival. It would be premature to attempt at this time to use these data to ascribe antibody formation to lymphocytes or other specific cellular constituents of these lead-shielded lymphatic tissues. The fact that the irradiated lymphatic tissue of the animals with either spleen or appendix shielding regenerates more rapidly than in animals without lead shielding may be as important in the achievement of high titers as the fact that an intact spleen or appendix is present.

The originally shielded spleen may be removed at 3 or 4 days after irradiation (2 or 3 days after antigen injection) or the originally shielded appendix may be removed 5 days after irradiation (4 days after antigen injection) and antibody formation still proceeds. These facts may indicate that the intact

(lead-shielded) appendix or spleen initiates the process of antibody formation or makes it possible for the process to be initiated. By 4 to 5 days regeneration in these animals at sites other than the originally protected tissue is perhaps far enough advanced to take over antibody production, or antigen is still available to initiate antibody formation as the irradiated tissue regenerates. It will be interesting to determine how soon after antigen administration these lead-shielded tissues must be removed to prevent the attainment of significant antibody titers. It will also be interesting to determine the increment of the total-body dose which must be given to the exteriorized spleen or appendix to suppress antibody formation such as occurs without spleen or appendix shielding.

In a recent publication Rowley(12) presents evidence that after splenectomy rats fail to respond with high circulating antibody titers if the dosage of antigen is small. In the same publication he reviews other work supporting his findings. Probably no real discrepancy exists between the splenectomy work referred to above and the findings reported in this present communication since the conditions of the experiments were so different.

*Conclusions.* These experiments corroborate Hektoen's original classic findings that antibody formation is suppressed by total-body X radiation. It has been demonstrated, in addition, that if the spleen or appendix of the rabbit is protected by lead shielding during total-body irradiation, the capacity to produce antibodies to an injected particulate antigen is retained to a marked degree even though lymphatic tissue elsewhere in the body is temporarily destroyed.

12. Rowley, D. A., *J. Immunol.*, 1950, v64, 289.

## Labeled Methionine as an Indicator of Protein formation in Children with Lipoid Nephrosis.\* (18128)

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Hypoproteinemia and proteinuria are constant findings in patients presenting the nephrotic syndrome. Whether the proteinuria is the sole factor responsible for the hypoproteinemia or whether a faulty mechanism of protein synthesis is also involved is a question that has evoked considerable speculation(1). The amount of protein excreted daily by these patients may be as much as 30 g but is usually not more than 5 to 10 g(2,3). It has been estimated that normal man on an adequate diet should be capable of forming 55 g of protein per day in excess of metabolic needs(4). From these considerations it would appear, *a priori*, that a faulty protein metabolism might well be implicated as a contributing cause of the hypoproteinemia in lipoid nephrosis. However, there is insufficient evidence available to prove this point unequivocally.

The demonstration that methionine labeled with radioactive sulfur is incorporated into body proteins and that the rate of incorporation may be taken as an indicator of the rate of protein synthesis(5,6) provided the opportunity for a new approach to the question

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3. Peters, J. P., and Bulger, H. A., *Arch. Int. Med.*, 1926, v37, 153.

4. Tui, C., Bartter, F. C., Wright, A. M., and Holt, R. B., *J. Am. Med. Assn.*, 1944, v124, 331.

5. Tarver, H., and Schmidt, C. L. A., *J. Biol. Chem.*, 1942, v146, 69.

6. Tarver, H., and Reinhardt, W. O., *J. Biol. Chem.*, 1947, v167, 395.

of the existence of an impaired mechanism of serum protein synthesis in the nephrotic patient. In the present study the rate of serum protein formation by nephrotic patients, as determined by this method, was compared with that of control subjects of a corresponding age group.

*Materials and methods.* The experimental subjects employed in these studies were 4 children whose illness was diagnosed as lipoid nephrosis on the basis of their having anasarca, ascites, albuminuria, hypoproteinemia and hyperlipidemia without arterial hypertension or hematuria. The control subjects were 2 children who were free from proteinuria and any demonstrable metabolic abnormalities related to serum protein formation. One of them, K. B., a 6-year-old girl, was physically normal but was subject to convulsive seizures of post-traumatic origin; the other, L. S., was a mentally retarded but physically healthy boy 1½ years of age.

Methionine, labeled with S<sup>35</sup> was administered intravenously in 20 ml of 0.85% NaCl solution. Since the same lot of radioactive methionine had to be used over a period of several months the absolute amount injected (4.5 to 24 mg) varied according to the radioactivity as determined at the time of each experiment. Blood specimens were obtained for analysis 5 hours and again 24 hours later. Total serum protein sulfur content and radioactivity were determined by the methods employed by Tarver and Schmidt(5) with minor modifications, including the employment of a "thick-layer" counting technic.

*Results and discussion.* The results are expressed in terms of the "biological concentration coefficient" (B.C.C.), as recently proposed by Schulman *et al.*(7,8), an expres-

7. Schulman, J. Jr., Falkenheim, M., and Gray, S. J., *J. Clin. Invest.*, 1949, v28, 66.

8. Schulman, J. Jr., and Falkenheim, M., *Nucleonics*, 1948, 3, No. 4, 13.

## PROTEIN SYNTHESIS IN LIPOID NEPHROSIS

TABLE I. Effects of Injected Radioactive Methionine in Nephrotic and Control Subjects.

Subject	K.B.	L.S.	J.M.	R.M.	D.F.	P.S.
Diagnosis	Control	Control	Nephrosis	Nephrosis	Nephrosis	Nephrosis
Age (yr)	6	1.5	2	3.5	7	3.5
Sex	F	M	F	M	M	M
Wt in kg	20.60	7.10	17.85	15.25	27.40	23.10
Total serum protein (g per 100 ml)	7.10	4.94	3.47	3.44	3.00	2.86
Dose of radioactivity inj. (counts per min.)	618,670	618,670	618,670	643,150	311,780	477,750
			5 hr after inj.			
Counts per min. in 5 ml serum	142.2	327.0	389.0	273.0	56.4	217.0
Millimoles sulfur in 5 ml serum	0.148	0.126	0.067	0.071	0.065	0.063
BCC*	3199	2978	16,752	9,117	7,625	16,654
			24 hr after inj.			
Counts per min. in 5 ml serum	127.9	251.4	269.7	223.7	—	216.5
Millimoles sulfur in 5 ml serum	0.139	0.104	0.057	0.069	—	0.064
BCC*	3,063	2,774	13,642	7,687	—	16,356

$$* \text{ Biological concentration coefficient (B.C.C.)} = \frac{(\text{e.p.m./millimol}) \times 100}{\text{e.p.m. administered/g body wt}}$$

sion permitting meaningful comparisons of the rate of incorporation of radioactive material into the serum proteins of individuals differing in weights and receiving varying dosages of radioactivity. The data obtained are summarized in Table I. From these data it may be seen that the B.C.C. values of the two control subjects are in remarkably close agreement, although the patients differ considerably in age, weight, and total serum protein concentration. In striking contrast to these values are the distinctly higher ones exhibited by each of the four nephrotic patients, values ranging from 2.5 to 5.5 times those of the control subjects.

It would appear from these results that the rate of incorporation of methionine into the serum proteins of nephrotic patients is greater rather than less than that of control subjects. The significance of this surprising finding is not immediately apparent. Superficially it would appear to indicate that there is no inadequacy of protein formation in nephrotic patients. However, it may be inferred that, whereas only the normal rate of production of serum proteins has been measured in the

control subjects, under the conditions of these experiments the maximal rate of their production has been measured in the nephrotic patients because of the stimulus afforded to such production by the inordinately low levels of protein in their serums. It must be considered also that the body proteins are in a state of dynamic equilibrium. Therefore, an increased concentration of radioactive materials in the serum protein need not necessarily reflect an increased rate of serum protein formation but might indicate a shift to the right of the equilibrium expressed by the equation, tissue protein = plasma protein. Furthermore, it seems possible that the presence of edema might cause spuriously high values of B.C.C. but it hardly seems probable that such errors could be of an order of magnitude that would explain the disparity observed between the nephrotic patients and the control subjects. Further investigations will be necessary to clarify these points.

*Summary.* 1. The rate of serum protein formation as indicated by the incorporation of methionine labeled with  $S^{35}$  was determined in 4 patients with lipoid nephrosis and in two

control subjects. 2. The rate of serum protein formation thus determined would appear to be greater in the nephrotic patients

than in the control subjects.

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## Effects of Hypo- and Hyperthyroidism in Rats and Mice on Ovarian Response to Equine Gonadotrophin.\* (18129)

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The effects of thiouracil and thyroprotein on the response of the testes and seminal vesicles of young rats and mice to a constant dose of pregnant mares' serum has recently been reported(1). It was found that thiouracil increased the gonadal response to PMS in rats but decreased it in mice. Thyroprotein decreased the gonadal response to PMS in rats but increased it in mice. Sex differences have been found in the normal thyroid secretion rate of male and female rats and mice(2,3). Other differences between the sexes may also modify the end-organ responses of animals to hormonal stimuli. It was considered of interest, therefore, to determine whether the gonadal response of female rats and mice to PMS would be altered in the same directions by thiouracil or thyroprotein as previously found in males of the two species.

**Methods.** Young female albino rats (Michigan State College strain) and mice (Rockland strain) were used in these experiments. The rats averaged 40 to 50 g in weight at the beginning and 80 to 100 g at the end of

the experiments. The mice weighed 8 to 12 g at the beginning and 17 to 21 g at the end of each experiment. The animals were housed in an air-conditioned room with a mean temperature of 74°F. Hyperthyroidism was induced in the rats and mice by incorporating thyroprotein† in the ration in concentrations of 0.02 to 1.28%, and feeding for 10 days. In one series of rats hyperthyroidism was produced by injecting d, l-thyroxine subcutaneously for 10 days in doses equivalent to 1.5 to 12 times their approximate normal thyroid secretion rate. Hypothyroidism was induced in the rats and mice respectively by feeding 0.1% and 0.2% thiouracil. The degree of hypothyroidism was controlled by feeding the thiouracil for 4 to 20 day periods. The animals which were to receive thiouracil for the longer periods were started at lower body weights than the controls in order to reach approximately the same body weights at the end of each experiment.

During the last 4 days of each experimental period, the rats were each injected subcutaneously with a constant dose of one Cortland-Nelson unit (20 I.U.) of pregnant mares' serum ("Gonadogen")§ and each mouse with one half of this dose. On the day of autopsy, the ovaries were removed and weighed on a Roller-Smith balance. The weights of the ovaries were calculated on a 100 g body weight basis.

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† Presented by the senior author in partial fulfillment of the degree of Master of Science at Michigan State College. Thanks are due Dr. E. P. Reineke for his helpful suggestions during the course of these experiments.

1. Meites, J., and Chandrashaker, B., *Endocrinology*, 1949, v44, 368.

2. Monroe, R. A., and Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bull.*, 1946, 403.

3. Hurst, V., and Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bull.*, 1948, 417.

‡ Thyroprotein ("Protamone") was made available through the courtesy of Dr. W. R. Graham, Jr. of Cerophyl Labs, Inc., Kansas City, Mo.

§ Kindly supplied by Dr. J. Lavere Davidson, Department of Veterinary Medicine, The Upjohn Co., Kalamazoo, Mich.

TABLE I. Effects of Thyroxine, Thyroprotein, and Thiouracil on Ovarian Response to PMS in Rats and Mice.

Species	No. of animals	Treatment		Avg wt of ovaries, 100 g body wt
		Was PMS given?	Other	
Rat	6	No	None	25.2 ± 0.9*
"	6	Yes	"	99.0 ± 4.6
"	12	"	6.9- 9.2 γ thyroxine	71.5 ± 2.7- 69.6 ± 1.8
"	18	"	18.4-55.2 γ "	57.7 ± 0.8- 41.7 ± 1.3
"	11	"	.04-.08% thyroprotein	66.6 ± 1.5- 63.9 ± 1.2
"	23	"	.16-32% "	59.7 ± 1.4- 49.7 ± 1.7
"	12	"	4 days thiouracil	154.0 ± 3.1-161.8 ± 2.4
"	12	"	7 "	183.3 ± 2.1-189.4 ± 2.6
"	16	"	10-15 days thiouracil	112.4 ± 1.7-104.7 ± 1.9
"	12	"	20 "	65.3 ± 1.3- 66.2 ± 2.6
Mouse	14	No	None	27.8 ± 1.1
"	7	Yes	"	41.5 ± 1.3
"	14	"	.02-04% thyroprotein	41.1 ± 1.3- 51.1 ± 1.5
"	14	"	.08-16%	52.3 ± 2.9- 55.7 ± 2.5

$$* \text{ Standard error of mean} = \sqrt{\frac{\sum d^2}{n(n-1)}}$$

**Results.** The data are summarized in Table I. Many of the groups of rats and mice, which averaged 6 and 7 per group respectively, have been combined in the table whenever the trend of the results was in the same direction. In the rats the administration of PMS alone increased the average weight of the ovaries from  $25.2 \pm 0.9$  to  $99.0 \pm 4.6$  mg. Both thyroxine and thyroprotein drastically reduced the response of the ovaries to PMS. The largest doses of thyroxine or thyroprotein given reduced the ovarian response to PMS approximately by half when compared with the group receiving PMS alone.

Thiouracil greatly increased the response of the ovaries of the rats to PMS when given for 4, 7, 10 and 15 day periods. The 7 day treatment with thiouracil increased the weight of the ovaries to twice that elicited by PMS alone. When given for 20 days, thiouracil effected a significant reduction in ovarian response to PMS in 2 groups of rats. Apparently, an extreme degree of hypothyroidism in female rats is not favorable to gonadotrophic action on the ovaries.

In the mice the injection of PMS alone increased the average weight of the ovaries from  $27.8 \pm 1.1$  to  $41.5 \pm 1.3$  mg. Thyroprotein significantly increased the response of the ovaries to PMS, with the largest dose, 0.16%, producing the greatest increase. It

will be noted that this effect of thyroprotein in the mice is just opposite to that obtained in the rats. Several groups of mice were treated with thiouracil, but the results were so inconclusive that they are not presented here.

**Discussion.** The results of these experiments in female rats and mice are in general agreement with the findings of Meites and Chandrashekar in male rats and mice(1). The one notable exception was that feeding thiouracil for 20 days reduced the action of the gonadotrophin in the female but not in the male rats. Feeding thiouracil for 4 to 15 days increased the gonadal response to PMS in rats of both sexes. It is believed that the possible effects of thiouracil or thyroprotein on the secretion of gonadotrophins by the animal's own pituitary can be eliminated as an important factor in these experiments. It was shown previously that these substances alone had no apparent effects on testes and seminal vesicle weights when given for 10 day periods to immature male rats and mice(1).

These data indicate that insofar as the gonadal response to PMS is concerned, young rats of both sexes secrete more than an optimal amount of thyroid hormone while young mice of both sexes secrete less than an optimal amount. These differences in thyroid status probably account for other anomalous

reactions to hormonal stimuli observed in the two species. Thus, thiouracil has been shown to decrease body growth(3) and mammary growth(4,5) in mice, but increase body growth(6) and mammary growth(5,7) in rats. Thyroid administration has been demonstrated to increase body growth(8) and mammary growth(9) in mice, but decrease body growth (8) in rats. It is not definitely known how thyroidal substances exert their modifying influences on the reaction of the gonads of rats and mice to PMS. A recent report by Warner and Meyer(10) indicates that in rats the administration of thyroxine probably acts directly on the ovaries to inhibit their reaction

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5. Trentin, J. J., Hurst, V., and Turner, C. W., *PROC. SOC. EXP. BIOL. AND MED.*, 1948, v67, 461.
6. Astwood, E. B., *The Harvey Lectures*, N. Y. Acad. of Med., Science Press Printing Co., Lancaster, Pa., 1945.
7. Johnston, R. F., and Smithcors, F., *Endocrinology*, 1948, v43, 193.
8. Koger, M., and Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bull.*, 1943, 377.
9. Mixner, J. P., and Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bull.*, 1943, 378.
10. Warner, E. D., and Meyer, R. K., *Endocrinology*, 1949, v45, 33.

to endogenous gonadotrophins.

*Summary.* The effects of experimentally induced hypo- and hyperthyroidism on the ovarian response to pregnant mares' serum were determined in immature rats and mice by administering thiouracil for 4 to 20 days and thyroxine or thyroprotein for 10 days. A constant dose of PMS was injected into each animal during the last 4 days of each experiment and the increase in ovarian weight was measured. It was found in rats that all doses of thyroprotein or thyroxine significantly decreased the action of the gonadotrophin on the ovaries, while in mice thyroprotein significantly increased the ovarian response to PMS. When thiouracil was fed to rats for 4, 7, 10 and 15 day periods, there were significant increases in ovarian response to PMS, but when fed for 20 days thiouracil reduced the ovarian response to PMS. These data on young female rats and mice corroborate the results previously obtained in young male rats and mice. It is suggested that insofar as the ovarian response to PMS is concerned, young rats of both sexes secrete more and young mice less than an optimal amount of thyroid hormone.

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## Effect of 5-Amino-7-Hydroxy-1H-v-Triazolo (d) Pyrimidine on Growth and Development of the Chick Embryo.\* (18130)

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In 1949, Kidder and his associates(1) reported that the growth of the protozoan, *Tetrahymena gelii*, and certain mammalian tumors was inhibited by the guanine analogue, 5-amino-7-hydroxy-1H-v-triazolo (d) pyrimidine (guanazolo).† Other investigators have studied the effect of this agent on a wider

range of neoplasms and considerable variation has been observed in the response of different tumors to it(2-4).

The purpose of this communication is to

† The alternate designation, "8-azaguanine", has been suggested for this compound(3).

2. Gellhorn, A., Engelman, M., Shapiro, D., Graff, S., and Gillespie, H., *Cancer Res.*, 1950, v10, 170.

3. Sugiura, K., Hitchings, G. H., Cavalieri, L. F., and Stock, C. C., *Cancer Res.*, 1950, v10, 178.

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\* This study was supported by a grant from The Charles F. Spang Foundation, Pittsburgh, Pa.

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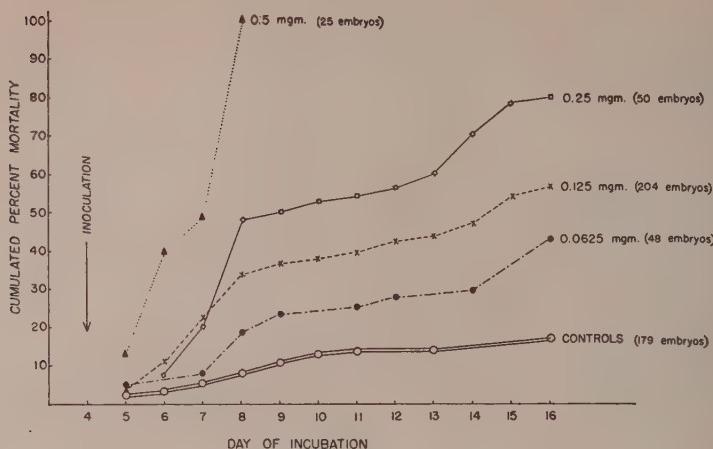


FIG. 1.

Cumulated percent mortality of chick embryos at intervals following yolk sac inoculation with different doses of guanazolo.

present observations on the toxicity of guanazolo for the chick embryo. This investigation was undertaken after it was noted in preliminary studies(5) that even small quantities of this compound affected embryos, in contrast to the reported absence of effect upon the host in studies in mice(1).

*Methods.* Fertile white leghorn eggs were incubated at 38.5°C and a relative humidity of 85%. Test solutions in 0.1 cc or 0.2 cc volumes were injected into the yolk sac by introducing a 1 $\frac{1}{4}$  inch, 23 gauge needle

through a hole in the blunt end of the egg. Guanazolo<sup>†</sup> solutions were prepared by dissolving 100 mg of the chemical in 2.0 cc of 1N NaOH and then diluting in distilled water to the desired concentration. Eggs were candled daily; dead embryos were removed, weighed, and examined. Experiments were usually terminated when the embryos were 16 days of age. All surviving embryos were sacrificed, weighed, and examined.

*Results.* In preliminary observations, made in the course of studies on effect upon virus

TABLE I. Weight Averages and Incidence of Abnormalities in 16-day-old Chick Embryos Inoculated on 4th Day of Incubation with Guanazolo or Various Doses of Guanine HCl plus Guanazolo.

Group	Inoculum (in .1 or .2 cc)	No. embryos	Avg wt, g	Range, g	Embryos showing abnormalities	
					No.	%
1	Controls ( $H_2O$ )	76	12.8	8.4-16.1	2	2.6
2	Guanazolo (.125 mg)	66	9.6	4.0-14.4	20	30.3
3	Guanazolo (.125 mg) + Guanine HCl (4.0-8.0 mg)	28	11.3	8.9-14.0	1	3.5
4	Guanazolo (.125 mg) + Guanine HCl (2 mg)	41	11.5	6.0-15.7	4	9.5
5	Guanazolo (.125 mg) + Guanine HCl (.5-1 mg)	47	12.0	6.0-15.6	6	13.7
6	Guanine HCl (8 mg)	26	12.2	8.5-15.2	0	0

5. Youngner, J. S., and Salk, Jonas E., *Cancer Res.*, 1950, v10, 250.

† The guanazolo used was furnished by the Ameri-

can Cyanamid Co., Lederle Laboratories Division, through the courtesy of Dr. J. M. Ruegsegger.

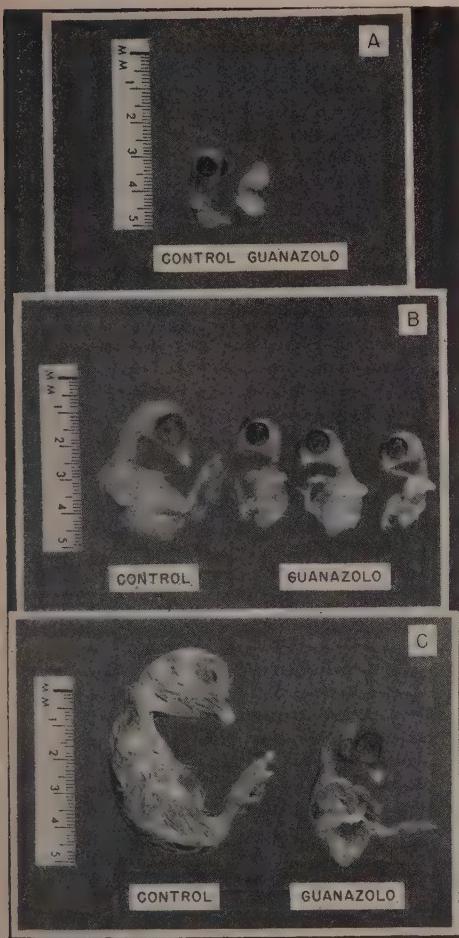


FIG. 2.

Appearance of chick embryos treated on 4th day of incubation with 0.125 mg of guanazolo and sacrificed at various intervals. Comparison with control embryos of similar ages. A—8th day of incubation; B—14th day of incubation; C—16th day of incubation.

multiplication, 10-day-old embryonated eggs were inoculated with guanazolo introduced into the allantoic sac or the yolk sac. Only in the latter instance did slight inhibition of growth occur. Deaths were noted at higher dosage levels (0.5 mg/egg). Corresponding dosages given allantoically were less toxic. Younger embryos were found to be more sensitive to the action of guanazolo, and in the majority of experiments here reported

inoculations were made on the 4th day of development. Embryos younger than 4 days of age did not survive toxic doses long enough for developmental abnormalities to occur.

The increased mortality resulting from injections of 0.1 cc of solutions containing different amounts of guanazolo given into the yolk sac of 4-day-old embryos is illustrated in Fig. 1. As can be seen from this graph, the mortality by the 16th day of incubation produced by 0.5, 0.25, 0.125 and 0.0625 mg doses of the drug was 100%, 80%, 56% and 43% respectively. The LD<sub>50</sub> was approximately 0.125 mg/egg. The control inoculum consisted of sterile distilled water. The small amount of alkali present in the different guanazolo concentrations employed was found to have no effect on growth or development when compared with untreated controls. This is not surprising since it has been found that normal yolk, early in development, is acid in reaction. In addition, the strong buffering action of the yolk and dilution of the inoculum would preclude significant changes in pH.

Surviving embryos at the 16th day of incubation were much smaller than the controls of corresponding age (Table I, Groups 1 and 2), and they exhibited a higher rate of developmental abnormalities. The abnormalities found were restriction both of vascularization and development of the chorioallantoic membrane, abnormal beaks, edema of the skin and peritoneal cavity, exteriorization of viscera, deformed extremities, inhibition of feather formation, and hemorrhage. Photographs illustrating some of these changes in treated embryos at different stages of development are shown in Fig. 2.

In order to determine whether the effects of guanazolo in chick embryos were the result of a generalized toxic action or, more specifically, an interference with normal guanine metabolism, the influence of guanine HCl on guanazolo inhibition was studied. Guanine HCl was dissolved in 1N NaOH (80 mg/cc) and desired concentrations prepared by diluting with sterile distilled water. Alkali alone, in quantities corresponding to that inoculated with guanine, had no effect on growth or development when compared

## GUANAZOLO, EFFECT ON CHICK EMBRYOS

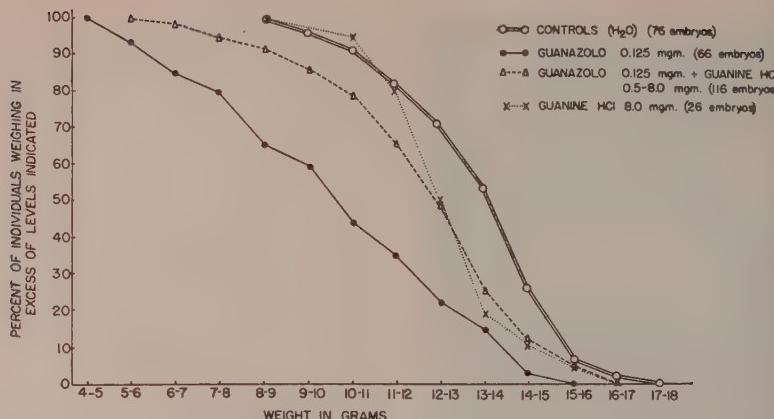


FIG. 3.

Comparison of weight distribution of 16-day-old sacrificed embryos treated on the 4th day of incubation with guanazolo alone, with guanine HCl alone, or with guanazolo plus guanine HCl. Weights plotted cumulatively as percent of individuals weighing in excess of indicated levels.

with untreated control embryos. Doses of guanine HCl ranging from 0.5 to 8 mg were injected into the yolk sacs of 4-day-old embryos together with 0.125 mg of guanazolo. As controls, groups of embryos were inoculated either with guanazolo or guanine HCl. Evidence of interference with guanazolo inhibition was obtained with all of the doses of guanine HCl employed. As can be seen in Table I, embryos that received guanine HCl plus guanazolo on the 4th day of incubation and were sacrificed on the 16th day of incubation (Groups 3, 4 and 5) were comparable in weight to the controls (Group 1). The distribution of weights of embryos inoculated with guanazolo alone (Group 2) was significantly lower.

In Fig. 3 is shown the weight distributions of individual embryos in the different groups. The points on the curve represent the percent of individuals in each group weighing in excess of the levels indicated. All embryos that had been inoculated with guanazolo plus guanine HCl were grouped together, regardless of the dose of guanine HCl. The proximity to that of the control of the curve for embryos given guanine plus guanazolo, and the relationship of both to the curve for embryos treated with guanazolo alone, suggests that guanine HCl, when injected together with guanazolo, interfered with the growth

inhibiting effect of the latter. In addition, developmental abnormalities were less frequent when guanine HCl was injected along with guanazolo (Table I).

**Discussion.** From the results presented it is clear that 5-amino-7-hydroxy-1H-v-triazolo (d) pyrimidine (guanazolo) even in very low concentration, has a profound effect on the growth and development of the chick embryo. The inhibition of the guanazolo effect by guanine HCl suggests that the chick embryo requires guanine but may not be able to synthesize this purine. These findings suggest a similarity in the nucleic acid metabolism of the chick embryo, *Tetrahymena*, and certain mammalian tumors. There are many indications of similarities in various metabolic systems in embryonic and tumor tissues(6) and the results reported here may indicate still another relationship. The specificity of the guanazolo effect is being tested further with adenine and embryos showing the effects of guanazolo treatment are being studied histologically.

**Summary.** The guanine analogue, 5-amino-7 - hydroxy - 1H - v - triazolo (d) pyrimidine (guanazolo), which has been reported to be relatively non-toxic for adult mice, was found to be toxic for the developing chick embryo.

This was manifested by a high mortality rate, inhibition of growth, and the occurrence of developmental abnormalities. The toxic effects of guanazolo were inhibited by the simultaneous injection of guanine HCl.

The technical assistance provided by Miss Doris M. Larsen in the beginning of this study is gratefully acknowledged.

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### Administration of the Hyperglycemic-Glycogenolytic Factor of the Pancreas to Non-Anesthetized and Anesthetized Subjects. (18131)

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That a hyperglycemic property may be present in pancreatic extracts and in commercial preparations of insulin has been recognized for some time. The recent preparation of a relatively purified hyperglycemic-glycogenolytic factor (HGF) from the pancreas and from gastric mucosa by Sutherland and DeDuve(1) and the demonstration of consistently reproducible *in vitro* and *in vivo* effects, have led to speculations concerning the possible role of such a factor in the regulation of carbohydrate metabolism in man, and particularly in the genesis of human diabetes. Nevertheless, Weisberg, Friedman and Levine(2) after summarizing the pertinent literature and from their own observations concluded that "as of the present, there has been no clear cut demonstration to prove that HGF is a hormone and/or that it is secreted by the alpha cell or the argentophil cells."

In view of the frequent presence of HGF in commercial preparations of insulin utilized in the treatment of diabetes mellitus in man, it was deemed advisable to assay the effects of the administration of a preparation rich in HGF to man. Since anesthesia has been shown to enhance the effect of the administration of

HGF to rabbits(2), the influence of this factor in men, with and without anesthesia was determined. It was deemed pertinent also to compare the response in man with that of other species.

*Method.* A preparation of insulin which was relatively rich in hyperglycemic factor was prepared by one of us (E.D.C.). It is estimated that this preparation contained that amount of HGF as would be present in an equal weight of commercial insulin. Large numbers of assays with cats and other animals revealed that as little as 0.05 mg per kg body weight produced a marked rise in blood sugar. The effectiveness of this preparation was not impaired by filtration through an ultra-fine fritted Pyrex disc bacterial filter. Before each experiment, a fresh solution containing 0.5 mg HGF per ml acid water (pH 2.5) was prepared and sterilized by fritted-glass filtration. After a preliminary period of 20 to 30 minutes during which blood samples were drawn, 0.1 ml of the HGF solution per kg body weight was injected intravenously. Subsequent specimens of venous blood were withdrawn at five-minute intervals for thirty minutes. In some instances simultaneous arterial samples were drawn. The majority of the subjects were ambulatory patients on the psychiatric wards and varied from 17 to 53 years in age. In addition, 2 patients with diabetes of mild severity were tested. In 8 patients, the experiment was performed both without anesthesia and with anesthesia. Altogether, 21 experiments were

\* Post-doctorate Research Fellow of the National Institutes of Health.

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2. Weisberg, H. F., Caren, R., Huddlestun, B., and Levine, R., *Am. J. Physiol.*, 1949, v159, 98.

performed on 11 subjects. The patients were all partaking well of the regular hospital diet, and with one exception, were allowed to eat breakfast between 7:30 and 8:00 a.m. on the day of the procedure. Lunch was withheld, and the experiments started between 1:00 and 2:00 p.m. In those instances where anesthesia was induced, it was accomplished by the intravenous injection of a 2.5% sodium pentothal solution in sufficient quantity to produce a light to moderately deep anesthesia before the first blood sample was obtained. Additional injections of pentothal were administered as necessary in order to maintain the anesthesia. In the 8 patients in whom the experiment was performed both with and without anesthesia, the 2 tests were performed in from 24 to 72 hours apart, and the order was varied, so that 3 patients were anesthetized during the first of the 2 tests performed on them and 5 were anesthetized during the second test. The blood was collected into tubes containing heparin and fluoride and the Nelson modification of the Somogyi method(3) was used for the determination of the glucose concentration.

**Results.** The intravenous injection of as little as 0.05 mg of extract containing HGF per kg body weight resulted in a rapid rise in the blood sugar in various species as il-

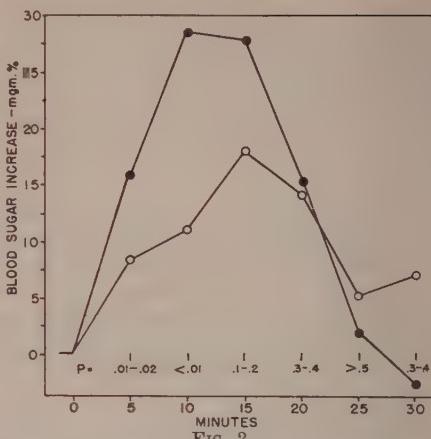


FIG. 2.  
Effect of anesthesia on action of HGF in man.  
○ = unanesthetized; ● = anesthetized.

lustrated in Fig. 1 and 2. The increase in venous blood sugar of the unanesthetized subjects varied from 6 mg % to 35 mg % with a mean of 19 mg %. Increasing the amount of extract administered did not increase the hyperglycemic effect in one patient on whom 3 experiments were performed without anesthesia nor in the many cats given from 0.05 to 0.5 mg per kg body weight.

In all 8 patients in whom the effect of administration of HGF was studied with and without the production of anesthesia, the increase in the blood sugar during anesthesia exceeded that observed in the same subjects without anesthesia, Fig. 2. Statistical analysis of the data revealed a significant difference between the effect of HGF only during the first ten minutes after the administration of the extract irrespective of the presence or absence of anesthesia. Thereafter there was no significant difference between the 2 groups.

In the 2 subjects with mild diabetes, one of whom had been fasted overnight after a high carbohydrate meal at 10 p.m. and who received an injection of 0.2 mg of the HGF per kg, the rise in blood sugar was 8 and 12 mg % respectively.

None of the patients demonstrated any untoward reaction which could be attributed to the preparation which was administered. In most instances the pulse rate was measured before, and at frequent intervals during the

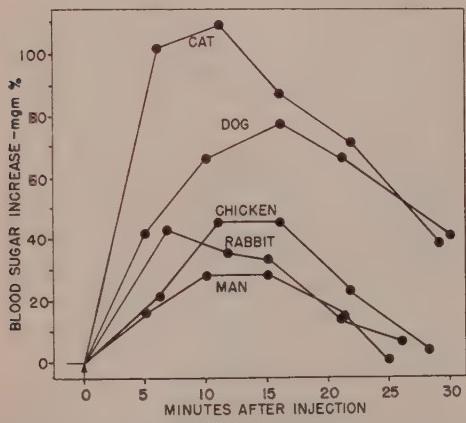


FIG. 1.  
Effect of HGF in various species. 0.05 mg HGF was administered intravenously after induction of anesthesia.

procedure and no significant changes were observed.

**Discussion.** The results described, demonstrate that a consistent hyperglycemic effect, variable in degree, can be produced in man by the intravenous injection of a potent preparation of hyperglycemic factor. This hyperglycemic effect is apparently increased by anesthesia as has been reported in animals by others(2).

The nature of the influence of anesthesia on the hyperglycemic effectiveness of this factor is not understood. An adequate explanation may lie in the well-established effect of anesthesia on increasing the rate of the peripheral circulation(4). Such an increase in the peripheral blood flow can be expected to reduce the arterio-venous difference in blood sugar. If that be the case, it may be anticipated that the rise in arterial blood sugar produced by the injection of HGF will be unaffected by anesthesia while the rise in the venous blood sugar will approximate that of the arterial blood sugar. In accord is the experiment in which simultaneous arterial and venous blood sugars were determined. Fig. 3 illustrates the data obtained in an anesthetized cat following the intravenous injection of HGF. Whereas the

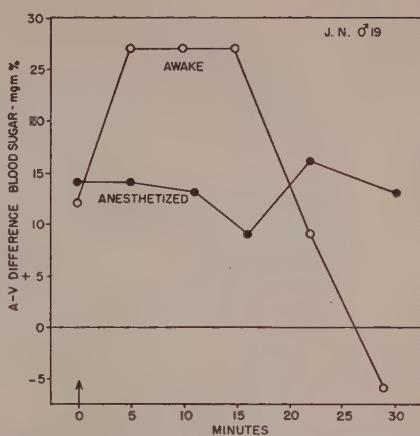


FIG. 4.  
Effect of HGF on arterial-venous blood glucose difference.

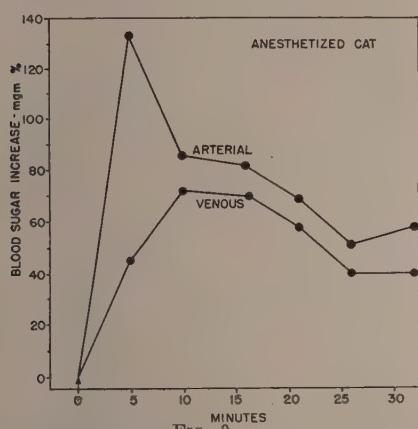


FIG. 3.  
Effect of HGF on arterial and venous blood of the anesthetized cat.

4. Abramson, D. I., *Vascular responses in the extremities of man in health and disease*, Univ. of Chicago Press, Chicago, Ill., 1944.

arterial blood glucose was increased by 105 mg per 100 cc, within 5 minutes, the concentration of glucose in the venous blood increased by only 43 mg within the same interval of time. Thereafter the glucose concentration of both the arterial and the venous blood approached similar levels. In one patient (J.N.), simultaneous capillary and venous blood specimens were obtained in experiments performed with and without anesthesia. In the experiment without anesthesia, the rise in venous blood sugar following injection of the HGF was 29 mg % while the rise in capillary blood sugar was 44 mg %; under anesthesia, the rise in venous sugar was 30 mg and that of the capillary sugar was 27 mg %. Thus, calculation of the A-V differences reveals a marked effect of the HGF injection in the absence of anesthesia and an insignificant effect in the presence of anesthesia (Fig. 4).

The preceding suggests that anesthesia does not affect the glycogenolytic action of the hyperglycemic factor of the pancreas. Since the effect of this factor in both anesthetized and unanesthetized men is relatively small, it can be postulated that the amount present in commercial insulin will produce negligible effects when administered to patients in diabetic coma.

**Summary.** The intravenous injection of a purified preparation of the hyperglycemic factor of the pancreas produces an increase in

the venous blood sugar of man. This response is variable in quantity and is not increased by increased dosage. The production of anesthesia enhances the increase in venous blood sugar produced by the hyperglycemic

factor. This effect of anesthesia is attributed to an increase in peripheral blood flow rather than to an augmented hyperglycemic effect.

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## Experimental Histoplasmosis in the White Rat.\* (18132)

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The true effectiveness of any substance of potential promise in the therapy of an infectious disease can be evaluated only in terms of its capacity to inhibit or kill the pathogenic microorganism in the tissues of an infected host. It is perhaps not unreasonable to suggest that lack of a standardized laboratory procedure for testing the *in vivo* activity of chemotherapeutic agents has been responsible for the delay in the development of an effective method of treatment of the systemic mycotic infections. With increasing recognition of the prevalence of these diseases has come an awareness of the necessity for some specific form of therapy, since the systemic mycoses, though not of common occurrence, have a high mortality rate. Extensive experience with histoplasmosis at the Vanderbilt University Hospital led us, a little more than a year ago, to undertake the development of a standardized technic for infecting animals with *Histoplasma capsulatum*. The widespread interest in histoplasmosis is reflected in the recent appearance of two articles on the experimental production of this fungous disease in animals. Campbell and Saslaw(1), and Howell, Kipkie and Bruyers(2), have been able to demonstrate

consistently successful infection of mice with *H. capsulatum*. The present paper is a report and discussion of the procedures we have developed for inducing generalized histoplasmosis in the white rat.

*Methods and experiments. Animals.* Since it was anticipated that certain drugs to be tested might have to be administered by gavage, mice were not used because of their small size. Guinea pigs had been shown (unpublished data) to be unsuitable because of a too great lack of uniformity in reaction to infection with *H. capsulatum*. White rats, of the Sherman strain, were tried and found to be reasonably satisfactory; healthy, white females, weighing  $125 \pm 8$  g were used throughout the experiments.

*Inoculum.* Preliminary experiments, over a period of a few months, with small groups of animals inoculated with equal numbers of organisms in each experiment, showed a great variation in mortality rate. The hypothesis was advanced, then proven correct, that even though the absolute numbers of organisms injected into the rats were identical, the number of *viable* organisms in the inoculum differed greatly. Accordingly, a rigid technic and schedule of transfer was instituted; under these conditions, transfer of cultures at three-day intervals produced a growth which, at the time of transfer, yielded a relatively constant percentage of viable organisms. After virulence tests on several strains of *H. capsulatum* obtained from different sources, V. U. strain No. 621,<sup>†</sup> which appeared more viru-

\* This work was aided in part by grants from the Institute of Paper Chemistry, Appleton, Wis., and the National Tuberculosis Association.

1. Campbell, C. C., and Saslaw, S., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 469.

2. Howell, A., Jr., Kipkie, G. F., and Bruyers, P. T., U. S. Public Health Rep., 1950, v65, 722.

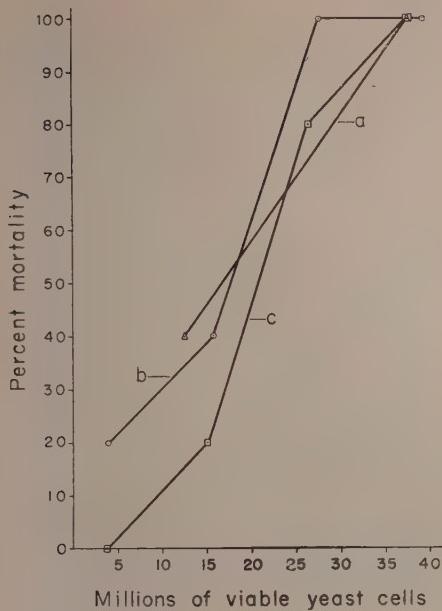


FIG. 1.

Relationship between number of viable yeast cells of *Histoplasma capsulatum* injected intravenously into rats and the percentage mortality obtained. Curves "a", "b", and "c" represent three different experiments carried out at several months intervals.

lent than the others, was chosen and used throughout the studies reported here.

Yeast cells from 3-day-old cultures were suspended in physiological saline, and adjusted photometrically to a standard density containing a known number of organisms. On the basis of the previously established probable percentage of viable organisms, suspensions of yeast cells were made up to contain the desired numbers of viable organisms per ml. (In each experiment, determination was made of the percentage of viable organisms in the standard suspension, and any deviation from the expected percentage taken into account in the final evaluation of dosage-mortality relationships.)

**Procedures.** Preliminary runs indicated that a 0% to 100% mortality prevailed when the number of viable organisms injected per ani-

mal was varied between 4 to 40 millions. Five rats were used for each of 4 different dosage levels of organisms; the animals were inoculated by tail vein with 1 ml of a suspension containing the calculated probable number of viable yeast cells. In view of the large number of organisms found necessary to effect a high mortality, it was felt desirable to inject a small group of animals with killed organisms. For this purpose 5 rats were injected intravenously with 1 ml containing the maximal number of viable organisms, which had been heat-killed. In another experiment, 5 rats were injected intraperitoneally, with the maximal number of organisms. All animals were observed carefully until death ensued, or until after four weeks had elapsed, after which interval of time the animals rarely succumbed.

**Results and discussion.** The results of 3 typical runs, carried out several months apart, are shown in Fig. 1. Curve "a", which has only 2 points since the 2 higher dosage levels also yielded 100% mortality, is given here because it represents the results obtained in one of the earliest runs, and shows good correspondence with curves "b" and "c", which represent experiments carried out at considerably later dates. The LD<sub>50</sub> for these 3 runs ranges between 17 and 21 million viable yeast cells per rat.

No effects, other than a transitory weight loss, were shown by the rats inoculated intravenously with maximum doses of heat-killed organisms, or intraperitoneally with the same doses of viable organisms.

All animals which succumbed to the infection showed a steady weight loss, amounting to about 30% of their original weight, by the time of death. Such animals also manifested an increasing lethargy, with markedly decreased intake of food and water. The interval between the time of inoculation and the time of death showed no constancy, either within or between groups, and averaged about 15 ± 6 days.

These results, which demonstrate that fatal experimental histoplasmosis may be regularly produced in rats by intravenous injection of the yeast cells of *H. capsulatum*, have one

† V.U. strain No. 621 was isolated from a 5-month-old infant who died with disseminated histoplasmosis in 1949.

or two points of interest which deserve comment.

Perhaps the most striking aspect of the experiments is the enormous number of viable organisms found necessary to establish a fatal infection. That the reaction was a true infection seems established by tissue sections from dead animals, which showed characteristic, developing, granulomatous areas containing numerous giant cells congested with the yeast cells of *H. capsulatum*. Furthermore, the injection of heat-killed organisms in maximal number was without significant pathological effect. Our results are in accord with Campbell and Saslaw(1), who used an inoculum which was about equivalent to ours, in terms of the number of organisms per gram of body weight necessary to produce death in the infected animal. (It may be noted, however, that Campbell and Saslaw used dosage levels based on the absolute numbers of yeast cells, stating that "it (is) impracticable to attempt to determine the actual number of viable cells.") Howell *et al.*(2) used intracerebral inoculation, and took into consideration the number of viable organisms in the inoculum. Interestingly enough, it may be calculated from their data that the intracerebral route of administration requires only 1/200 the number of viable yeast cells of *H. capsulatum* as does the intravenous route. It seems unlikely that a difference of this magnitude could be attributable to a

difference in virulence of the strains employed; probably it is a reflection of the difference in the capacity of the host's defense mechanisms to cope with invasion by these different routes. This point is further illustrated by the ineffectiveness of intraperitoneal injection in our tests, and in those of Campbell and Saslaw, who found it necessary to add gastric mucin in 5% concentration to the yeast cell suspension in order to obtain infection by the intraperitoneal route.

From the standpoint of using the infected rat for *in vivo* studies of fungicidal agents, it is perhaps unfortunate that a change from 0% to 100% mortality should occur with only a ten-fold increase in the number of viable organisms injected. This fact, together with the inevitable uncertainty regarding the percentage of viable organisms in an inoculum, makes it essential to employ more than one dosage level of viable organisms, in order to achieve good approximation to any particular desired percentage mortality in the control animals.

**Summary.** The yeast cell phase of *Histoplasma capsulatum*, when injected intravenously in suitable numbers, has been shown to yield a consistently reproducible fatal infection in the rat. The necessity for standardizing the inoculum on the basis of the number of viable organisms, rather than the absolute number of organisms, is emphasized.

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## Experimental Intraocular Infection of Guinea Pigs with Mumps Virus.\*† (18133)

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The virus of mumps is known to infect

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§ Presented in part at the 5th meeting of the Intermountain Branch of the Society of American Bacteriologists, Salt Lake City, Utah, October 8, 1949.

the human being(1), the monkey(2), and the chick embryo(3) with the production of complement fixing antibodies in man and monkeys. Previously, the guinea pig has been found to produce no complement fixing antibodies after intraocular injection of filtrate from infected parotid gland of the monkey(4).

This paper presents experiments on (a) the susceptibility to mumps virus of the ocular tissues of the guinea pig; (b) the recovery of mumps virus from the ocular tissue of guinea pigs after five successive passages; (c) the production of high complement fixing antibody titers after intraocular injection of mumps virus, and (d) the irregular production of complement fixing antibody after extraocular inoculation of mumps virus.

**Material and methods.** An embryo-adapted strain of mumps virus which had undergone 52 passages in embryos was employed in these experiments.<sup>||</sup> The virus pool was prepared by inoculating 0.1 ml of infected allantoic fluid into the allantoic sac of seven-day-old chick embryos. After 5 days of incubation at 35°C, the allantoic fluid was harvested, sealed in small pyrex tubes, and stored in a dry ice cabinet until used. In the allantoic sac of chick embryos this material had an average titer ( $ID_{50}$ ) of  $10^{9.3}$  50% infective doses per ml. Allantoic fluid collected from uninoculated 13-day embryos was employed as control for the intraocular injections. The guinea pigs employed in these experiments were young adult, albino males. From each guinea pig two samples of serum were collected. The first was taken immediately before mumps virus injection and the second 18 days later. Aqueous humor (0.02-0.05 ml) was withdrawn from the an-

terior chamber of the guinea pig eyes with a tuberculin syringe, fitted with a 27 gauge needle. The needle was introduced through the sclera about 1 mm from the limbus. The aqueous humor of the left eye was replaced with 0.05 ml of virus-infected allantoic fluid, and that of the right eye was replaced with 0.05 ml of normal allantoic fluid. Beef heart-infusion broth (pH 7.4) was used as the diluent.

In Exp. II and III the aqueous humor from the anterior chamber of the injected eyes was not cultured for bacteria. Emulsified ocular tissue from Exp. I was cultured on blood agar plates at 37°C for 48 hours. These cultures showed no bacteria under aerobic or anaerobic conditions. Certain specimens that had been frozen (some contained penicillin and streptomycin while others did not) were cultured for pleuropneumonia organisms with negative results. The pleuropneumonia culture technic included the use of aerobic and anaerobic methods with media containing horse serum and had proved adequate for the isolation of such organisms from rats, mice, and human prostatic secretion.<sup>¶</sup> Eyes that have been infected with bacteria are easily recognized by macroscopic inspection. In these experiments bacterial infections were rare, but to minimize the possibility of such infections both penicillin and streptomycin were employed, each in a concentration of 2.5 µg per ml(5). Kolmer's(6) method was employed for the determination of the levels in the sera of complement-fixing antibody against the mumps virus. The sera were preserved at -70°C or at 4°C so that all of the complement-fixation tests for each experiment could be performed simultaneously. Each serum was tested with normal allantoic fluid and complement for the detection of complement-fixing antibody against non-viral embryo material. The sera were inactivated by heating twice at 60°C for 20

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|| Originally supplied to one of us by Dr. J. F. Enders.

¶ Pleuropneumonia cultures were performed by Dr. John C. Nunemaker.

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6. Kolmer, J. A., and Boerner, F., *Approved Laboratory Technic*, 4th Ed., Appleton Century, 1945.

minutes to reduce anti-complementary effects. Titters were expressed as the final dilution of serum prior to adding the sensitized erythrocytes. End points were recorded as the highest dilution of serum which gave 3 or 4 + fixation of complement. For chicken red blood cell agglutination-inhibition tests, a modification of Salk's method for influenza was employed(7). Agglutination-inhibition tests for identification of the virus recovered from guinea pig ocular tissue were performed, using sera from both acute and convalescent human cases and from guinea pigs experimentally infected with mumps virus. The antigens used were (1) allantoic fluid infected with stock virus of the "Enders" strain, and (2) allantoic fluid infected with "recovered virus" from ocular tissues of guinea pigs.

Exp. I—Serial passage of mumps virus in guinea pig ocular tissue, employing a 10% pooled suspension of retina, uvea, cornea and lens for inoculum, has been performed. The passage suspensions were ground in a mortar with a pestle and centrifuged at 2000 r.p.m. for 5 minutes. The supernatant was passed serially every 48 hours until 7 passages were achieved. Three to 8 guinea pigs were employed in each passage. Convalescent sera collected from the 2 guinea pigs of passage I (injected with undiluted stock virus) and 1 of 3 guinea pigs of passage II showed complement-fixing antibodies in titers of 1:640, 1:1280 and 1:20, respectively, against the stock virus. The convalescent sera obtained from the guinea pigs in passages III, IV, V, VI and those injected with Seitz filtrate from which virus was recovered in embryonated eggs, failed to show complement-fixing antibodies against the stock virus.

Six guinea pigs were injected with suspensions from passage VI. Of the 6 animals, 2 were injected with pooled suspensions of corneas and 4 with pooled suspensions of retina, uvea and lens. The sclerae were discarded. The convalescent sera from 2 of the 4 guinea pigs in passage VII, injected with ocular tissue other than cornea, showed complement-fixing antibody titers of 1:160

TABLE I. Comparative Agglutination-Inhibition Titers Produced by Human and Guinea Pig Sera Against Stock and Recovered Virus.

Source of sera	Sera phase	Recovered virus	Stock virus
Human (LL)	Acute	32	32
	Conv.	2048	2048
	Acute	128	128
Human (NW)	Conv.	2048	2048
	Acute	<2	<2
	Conv.	1024	512
Guinea pig #390	Acute	16	16
	Conv.	2048	1024
Guinea pig #873			

and 1:320 against the stock virus, indicating infection with the adapted guinea pig virus. Pooled Seitz filtrate from ocular tissues of passage V with either VI or VII was injected into both eyes of 7 guinea pigs and into 8 seven-day-old embryonated eggs, according to the technic previously described(5).

On the 5th day after inoculation of the above filtrate, the pooled allantoic fluid collected from 7 of the 8 embryos caused hemagglutination of chicken red blood cells at a titer of 1024. The identity of the recovered virus subsequent to a second egg passage was established in 3 ways. First, comparative agglutination-inhibition tests were performed, employing pools of stock and recovered virus as antigens against acute and convalescent sera from human patients with mumps and from experimentally infected guinea pigs.

Results as presented in Table I show that the sera had comparable agglutination-inhibition titers against each virus. Employing the two viruses, convalescent human and guinea pig sera and complement fixation tests, comparable levels of complement fixing antibodies were demonstrated, indicating the serological relationship of the recovered virus to stock virus. The recovered virus was inoculated into the anterior chamber of the eyes of 5 guinea pigs. The inoculated eyes developed a mild corneal reaction. The convalescent sera from these guinea pigs with stock virus fixed complement and resulted in titers of 1:40-1:160. Complement fixation tests, for the detection of antibody formation in guinea

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TABLE II. Production of Complement-Fixing Antibodies and Corneal Opacity as a Result of Intraocular Injection of Mumps Virus.

Guinea pig No.*	Eye inj.	Dilution of inoculum†	Persistence of corneal opacity			Results of CF tests‡	
			24 hr	72 hr	18 days	Initial sera	Conv. sera
199	Rt.	A, $10^{-3}$	0	0	0	<10	80
	Lt.	V, $10^{-3}$	+	±	0		
200	Rt.	A, $10^{-3}$	0	0	0	<10	<10
	Lt.	V, $10^{-3}$	0	0	0		
201	Rt.	A, $10^{-2}$	0	0	0	<10	80
	Lt.	V, $10^{-2}$	0	±	0		
202	Rt.	A, $10^{-2}$	0	0	0	<10	320
	Lt.	V, $10^{-2}$	++	++++	++++		
203	Rt.	A, $10^{-1}$	0	0	0	<10	320
	Lt.	V, $10^{-1}$	++	++++	++++		
204	Rt.	A, $10^{-1}$	0	0	0	<10	320
	Lt.	V, $10^{-1}$	+++	++++	++++		
211	Rt.	A, $10^0$	0	0	0	<10	320
	Lt.	V, $10^0$	++	++	++++		
212	Rt.	A, $10^0$	0	0	0	<10	320
	Lt.	V, $10^0$	+++	++++	++++		

\* Eyes of guinea pigs (189-198) injected with virus dilution  $10^{-4}$  or higher, developed no corneal reaction while the initial and convalescent sera of these animals showed no complement-fixing antibodies in a dilution of 1:10, the lowest dilution tested.

† A = Dilution of 13-day normal allantoic fluid injected into right. V = Dilution of mumps virus-infected allantoic fluid injected into left eye.

‡ Normal allantoic fluid controls were negative in each instance of lowest serum dilution tested (1:10). Titers expressed as the denominator of the dilution.

pigs against non-viral material in allantoic fluid, proved to be negative in a dilution of 1:10, the lowest dilution of sera tested. This was found to be true also for the two experiments reported below.

Exp. II.—This experiment was performed to determine the relative amounts of injected stock mumps virus required to produce infection in the eye of the guinea pig as indicated by the corneal reaction and the subsequent appearance of complement-fixing antibodies in the convalescent sera of guinea pigs against the stock mumps virus. Mumps infected allantoic fluid, undiluted and in ten-fold dilutions through  $10^{-8}$ , was injected into the left eyes of eighteen guinea pigs, 2 animals being used for each dilution. The right eyes were injected in a similar manner with similarly diluted normal allantoic fluids. As may be seen in Table II, one of the two guinea pigs injected with virus dilution  $10^{-3}$ , as well as one of those two injected with virus dilution  $10^{-2}$ , and all of those receiving lower dilutions, developed complement fixing titers of 1:80-1:320. Guinea pigs injected with virus dilutions  $10^{-4}$  or higher, and one guinea

pig (No. 200) injected with virus dilution  $10^{-3}$  developed no complement fixing antibodies against the mumps virus. Guinea pigs injected with virus dilution  $10^{-2}$ , or lower, developed a severe corneal reaction with subsequent pannus that was still present in about the same degree 18 days later. The corneal reaction began to appear within 24 hours after the inoculation of material which contained relatively large amounts of virus. At 48-72 hours after injection the entire cornea was involved. When smaller amounts of virus were employed, the corneal reaction was transitory and the normal translucency returned to the cornea in 3 to 14 days. This has been observed previously in experiments not reported in this paper. A minimal corneal opacity that disappeared in 72 hours was seen in guinea pigs No. 201 ( $10^{-2}$  dilution) and No. 199 ( $10^{-3}$  dilution). These minimal corneal reactions were associated with complement-fixing antibody titers of 1:80. Four of the 8 left eyes injected with dilutions of virus beyond  $10^{-4}$  developed a transitory corneal reaction which disappeared within 24 hours. No complement-fixing anti-

body was demonstrated in these guinea pigs. The injection of the 16 right eyes with the various dilutions of normal allantoic fluid produced no corneal opacity.

Exp. III.—The comparative effectiveness of intraperitoneal, intraplantar space and intracardial virus injection for complement-fixing antibody production was studied. Three to five hundredths ml. of undiluted mumps virus-infected allantoic fluid was injected into the peritoneal cavity of 6 guinea pigs and into the plantar spaces of the hind foot of a similar number. Four animals were injected intracardially with 0.05 m. of the same fluid.

Of the 16 guinea pigs, one developed a complement-fixing antibody titer of 1:40, 5 of 1:20, 3 of 1:10, and 7 less than 1:10, the lowest dilution of sera tested.

*Discussion.* The characteristic corneal reaction that develops after the injection of relatively large amounts of mumps virus into the eyes is probably a result of the direct action of the virus itself on the endothelial cells of the inner surface of the cornea. The nature of this effect is being tested by experiments now under way. From the data shown in Table II, it is clear that relatively large amounts of embryo virus must be injected into the eye before the guinea pig can produce complement-fixing antibody. Subsequent to intraocular inoculation, 99% of the virus disappears from the eye within 48 hours, following which it grows or persists within the ocular tissues for at least 8 days(8).

The intraperitoneal, intraplantar space or intracardial inoculation of six to ten times the amount of mumps virus that is adequate for antibody production after intraocular injection has failed to produce complement-fixing antibodies, or resulted in low titers only. The explanation for this is probably related to failure of growth or limited growth of virus in extraocular guinea pig tissue.

The production of complement-fixing antibodies in the guinea pigs of passage II may be in part the result of the transfer of "residual" virus from passage I, 48 hours after inoculation. The failure of complement-fixing anti-

bodies to appear in the sera of guinea pigs of passages III, IV, V, VI, and of those injected with the Seitz filtrate pool against the stock virus is not known. In passage VII, 2 of 4 guinea pigs developed complement-fixing antibody titers of 1:160 and 1:320. This indicates that these guinea pigs developed a virus infection as a result of the ocular injection of passage VI. Mumps virus was recovered on two different occasions from a Seitz filtrate pool of passage V pooled with either passage VI or VII injected into 7-day-old embryonated eggs. It is unlikely that the virus recovered from the fifth passage was merely "residual" virus carried along from the first passage since 5 passages would produce about a 10,000 fold dilution of egg virus, which is beyond the range of infectivity for guinea pig ocular tissue as may be seen in Table II.

Additional studies have been planned to work out the inconsistencies which have occurred during the serial passage of virus in guinea pig eyes.

*Summary and Conclusions.* 1. Chick embryo-adapted mumps virus has been shown to infect the tissues of the guinea pig's eye after intraocular inoculation.

2. Mumps virus has been carried through 7 successive guinea pig passages by intraocular injection with the subsequent production of complement-fixing antibodies in the sera of guinea pigs in passages I, II and VII. A virus culturally and immunologically indistinguishable from the original virus has been recovered from ocular tissue of passages V, VI and VII by inoculation into chick embryos.

3. When doses of virus 6 to 10 times as large as those necessary to infect the anterior chamber were introduced into the plantar spaces, peritoneal cavity, or heart, antibody formation was low or absent as shown by titers of the complement fixing antibody in the convalescent sera.

4. The guinea pig appears to be a suitable animal for the study of mumps virus infections when inoculations are made into the anterior chamber of the eye.

8. Bolin, V. S., Bosma, J. F., and Newton, J. D., Unpublished data.

## Macrophage Content of Oil-Induced Peritoneal Exudate in Rats and Rabbits.\* (18134)

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(Introduced by P. B. Beeson.)

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The ease with which experimental tuberculosis can be produced in guinea pigs and rabbits indicates a low degree of natural resistance to tubercle bacilli. In contrast, it is extremely difficult to produce experimental tuberculosis in the rat. A high degree of natural resistance in the rat offers an unusual opportunity to investigate problems of host resistance in an animal which in general differs from man and other animals in regard to hypersensitivity and to antibody production. The cellular defense against tuberculosis has been extensively studied by Medlar(1), Sabin *et al.*(2), and Martin *et al.*(3). It has also been shown(3,4) that polymorphonuclear leukocytes ingest tubercle bacilli, but are themselves injured early in the process. The tissue macrophage is another phagocytic cell involved in the inflammatory reaction. This cell becomes prominent later than the polymorphonuclear leukocyte in the development of inflammation, and it is not uncommon to find tubercle bacilli and dead polymorphonuclear leukocytes within the cytoplasm of the mononuclear phagocytes.

Previous studies by Doan *et al.*(5), indicated that the predominant response of the host to foreign lipids was a mobilization of mononuclear phagocytes. With this knowl-

edge at hand, the present study was undertaken in an attempt to ascertain possible quantitative differences in macrophage response in species naturally susceptible and naturally resistant to tuberculosis.

*Materials and methods.* A modification of the method of Lucké *et al.*(6) was employed to obtain peritoneal exudates containing mononuclear cells. Albino rats, weighing between 150 to 250 g, and albino rabbits, weighing between 2,000 to 3,000 g, were injected intraperitoneally with 25 cc of sterile mineral oil per kilo of body weight. Forty-eight hours later the animals were sacrificed; a midline incision was made and 25 cc per kilo body weight of heparinized-Tyrode solution containing 10% homologous serum was introduced into the peritoneal cavity. The incision was closed with clamps and the fluid agitated in the abdominal cavity for five minutes. The milky emulsion formed was removed with a large sterile pipette and was placed in a separatory funnel for 15 minutes. The oil layer separated sharply and the aqueous phase was removed. White blood cell and differential counts were performed on the aqueous cell suspension in the usual manner. Wright's differential blood stain was used for studying the cells of the exudate.

*Results.* Table I reveals that the total white blood cell response of albino rats to intraperitoneal mineral oil injection is significantly greater than that of rabbits. A comparison of cell type obtained from these 2 species reveals a higher percentage of mononuclear cells in rabbit exudates. However, the total number of mononuclear cells (Table I, absolute values) is considerably greater in the rat.

In view of the difference of opinion con-

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\* Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the author are the result of his own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

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2. Sabin, F. R., Doan, C. A., and Forkner, C. E., *J. Exp. Med.*, (Supp. No. 3), 1930, v52, 1.

3. Martin, S. P., Pierce, C. H., Middlebrook, G., and Dubos, R. J., *J. Exp. Med.*, 1950, v41, 381.

4. Bloch, H., *Am. Rev. Tuberc.*, 1948, v58, 662.

5. Doan, C. A., Sabin, F. R., and Forkner, C. E., *J. Exp. Med.*, (Supp. No. 3), 1930, v52, 89.

TABLE I. Differential and White Blood Counts on Intraperitoneal Exudates of Albino Rats and Rabbits.

Rat No.	WBC/mm <sup>3</sup>	Poly	Lymph	Eos	Baso	Mono	Absolute mono count/mm <sup>3</sup>
1	20200	61	22	3	0	17	3433
2	15900	53	30	0	0	17	2703
3	12200	21	55	0	0	22	2684
4	30450	45	33	1	0	22	6650
5	30150	62	18	5	0	15	4520
6	5050	3	30	0	0	67	3350
7	8700	1	46	0	0	52	4540
8	10550	75	8	2	0	15	1650
9	9500	57	30	1	0	13	1235
10	6800	70	5	0	0	25	1700
11	15200	59	22	0	0	19	2890
12	13300	17	17	0	1	65	8620
13	11200	22	10	0	1	67	7500
14	7100	18	5	0	3	74	5230
15	4800	6	25	0	1	68	3260
Mean	13321						3930
S.D.	±8086						±2425
<hr/>							
Rabbit No.							
1	800	20	5	0	0	75	600
2	1100	46	9	3	0	42	462
3	550	22	33	0	0	55	310
4	550	23	12	0	0	65	357
5	1350	10	6	0	0	84	1130
6	650	70	4	0	0	26	269
7	900	45	5	0	0	50	450
8	700	56	2	0	0	42	294
9	700	45	.7	0	0	48	336
10	700	43	2	0	0	55	385
11	2800	31	7	0	0	62	1735
12	1100	34	6	0	0	60	655
13	1200	10	8	0	0	82	984
14	2500	26	10	0	0	64	1600
15	1000	56	10	0	0	34	340
Mean	1114						660
S.D.	±673.5						±473.3

cerning terminology of the large mononuclear cells, we have, for the purpose of this study, used the term "mononuclear phagocyte" to include the cells which have previously been described as "macrophages," "clastmatocyte," "histiocytes," and "monocytes." The several types of cells included in our category are illustrated in Fig. 1. It can be seen that

many of these cells have ingested the mineral oil.

**Discussion.** It is known that phagocytic cells similar to, if not identical with, those observed in this study are extremely important to the host in the process of natural resistance to certain infections. It is suggested that one mechanism responsible for the rat's natural resistance is this animal's ability to mobilize a larger number of mononuclear phagocytic cells than can be mobilized by less resistant animals. Further work to explore this hypothesis is in progress.

**Summary.** 1. Albino rats and rabbits were injected intraperitoneally with sterile mineral oil to produce peritoneal exudates.

2. Peritoneal exudates of rats contained greater numbers of white blood cells than rabbit exudates.

3. There was a significantly greater mononuclear phagocyte response to the same stimulus in rats than in rabbits. It can be postulated that this response may be related to the native resistance of the rat to tuberculosis.

We wish to thank Miss Mary Louise Wilcox for her technical assistance throughout this study.

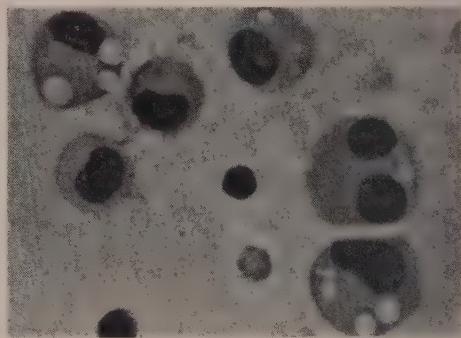


FIG. 1.  
Predominant cell type in peritoneal exudate.  
Note phagocytosis of oil droplets.

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## Blood Urethane Levels Achieved with Entero-Coated Tablets.\* (18135)

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(Introduced by Mario Stefanini.)

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The discovery of the growth suppressive power of the carbamic esters has raised this long known chemical group to the status of chemotherapeutic agents. Their failure to influence favorably advanced cases of malignant diseases in man has been more than compensated for by the discovery of their inhibitory action on white cell neoplasia. Peterson, Thomas, Haddow and Watkinson(1) first treated leukemias with ethyl carbamate (urethane). Since then, numerous articles have appeared in the literature dealing with the therapeutic results of this drug. In general, it appears to be more effective in chronic leukemias than in acute leukemia. More recently, Loge and Rundles(2) have called attention to the clinical effectiveness of urethane in the treatment of multiple myeloma.

Regulation of the dosage of urethane is difficult at times especially when the white count is normal or decreased. In leukemic states associated with high white cell count no such difficulty is encountered; the white blood cell count acting as a sensitive indicator. In these cases, a rapidly falling total white count or leucopenia calls for diminution or cessation of therapy. The inverse relationship between white count and dosage permits excellent control, (Fig. 1), and protects against the dangers of severe hepatic damage or aplasia of the bone marrow.

The utilization of urethane blood levels as a guide to dosage schedules suggested itself

\* This work was supported by grants in aid from the Charlton Fund and the American Cancer Society, Massachusetts Division.

† This work was completed during the tenure by the two authors of a Damon Runyon Clinical Research Fellowship, administered by the American Cancer Society.

1. Paterson, E., Aphthomas, I., Haddow, A. and Watkinson, J. M., *Lancet*, 1946, v200, 677.

2. Loge, J. P., Rundles, R. W., *Blood*, 1949, v4, 202.

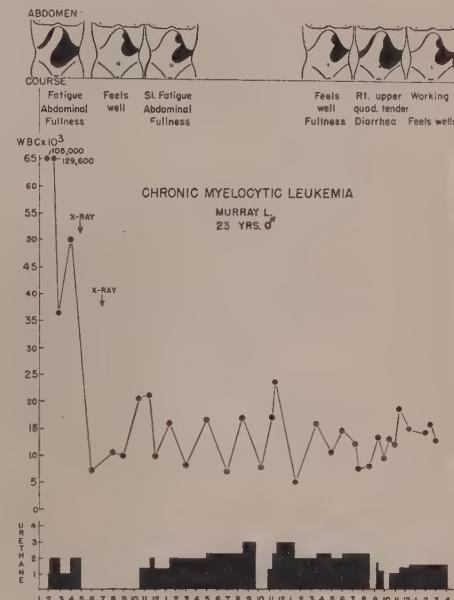


FIG. 1.

in those cases in which the white count offered no reliable guide. However, the very simplicity of the urethane molecule makes its quantitative estimation difficult. Archer, Chapman, Rhoden and Warren(3) have utilized the ethyl group as an aid in the measurement of the blood urethane level. The process consists essentially of alkaline hydrolysis of urethane to ethanol, carbon dioxide and water, the distillation and recovery of the ethanol and its quantitative measurement. This bears a direct relationship to the amount of urethane being estimated, which can then be calculated. These authors recognized that other volatile reducing substances may be formed during hydrolysis and

3. Archer, H. E., Chapman, L., Rhoden, E. and Warren, F., *Biochem. J.*, 1948, v42, 58.

performed blank determinations on samples in each case. Values cited were of 22 mg of urethane per 100 cc following a dosage of 3 g daily for 14 days and of 10 mg per 100 cc following 1 g daily for 50 days. The ingestion of 2 g in one dose resulted in a 28 mg% rise.

In the present study, an attempt was made to evaluate the relationship between dosage of drug and the blood urethane level in 10 patients all of which were under treatment for leukemia or multiple myeloma, using the method of Archer *et al.*(3). All patients had been on urethane therapy two weeks or longer. No effort was made to secure samples at set hours after the ingestion of the urethane tablets. However, all patients except one had samples drawn 3 to 4 hours after their usual divided maintenance dose. Urethane was administered in the form of enteric coated tablets (Lilly) 0.3 g each. The daily dosage, which ranged from 1 to 4 g was given in 3-4 divided doses usually directly after meals. Venous blood was used with heparin as an anticoagulant. A standard Folin-Wu Filtrate was made. All determinations were made on unstored samples within 2 hours after drawing the blood.

**Results.** Nineteen determinations were made on 10 patients. There appeared to be little correlation between the dosage schedule and the blood level obtained. For example, some patients receiving as much as 3 g of urethane daily showed either a low or even no measurable blood level (Fig. 2). In the group of patients receiving 2 g per day, blood levels ranged from 4 mg to 27 mg%. On a dosage scheduled twice this value, no higher blood levels were achieved.

**Discussion.** From our data it seems likely that urethane blood levels offer little aid in the guidance of therapy with this drug. Some cases having either no appreciable or very low urethane blood levels displayed well defined clinical improvement with or without leucocytic depression, testifying that the drug was being absorbed. More recently, using a more specific method for urethane determination but

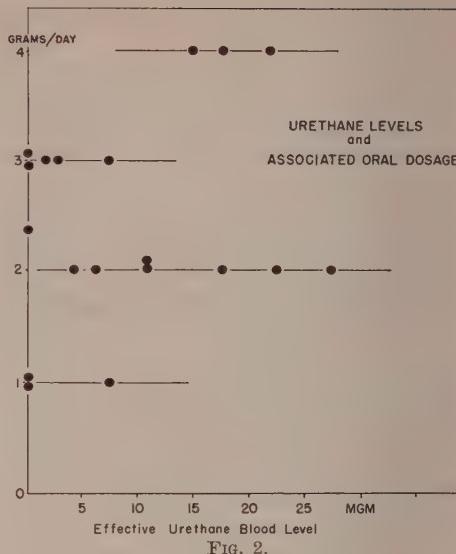


FIG. 2.

based on the same general principle(4), a similar lack of correlation was found in a limited number of determinations. It is possible that the enteric coating on the urethane tablets altered intestinal absorption so that therapeutically active but very low (blood) levels are obtained. In addition the unpredictability of intestinal absorption and the degree of tissue utilization of the drug(5) undoubtedly affect the reliability of blood urethane values as a therapeutic guide.

**Summary.** Blood urethane levels using the method of Archer *et al.* were performed on patients receiving enteric-coated urethane tablets for the treatment of leukemia and multiple myeloma. Blood levels ranging from 0 to 27 mg per 100 cc of blood were obtained. The correlation between dosage of the drug, blood level and clinical response was poor. This negates the value of such determination in the regulation of oral urethane therapy.

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5. Progress Report on the Chemotherapy of Leukemia, Southern Research Institute, 1949.

## Suppression of the Phenomenon of Local Tissue Reactivity by ACTH, Cortisone and Sodium Salicylate. (18136)

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It was recently reported that ACTH is capable of suppressing the phenomenon of local tissue reactivity, when administered intramuscularly 2 hours prior to the provocative injection of meningococcus culture filtrate(1). The experiments embodied in the following paper serve to extend these observations.

*Experimental. Substances failing to inhibit or modify the phenomenon of local tissue reactivity to bacterial filtrates.* The interpretation of the results of the present studies is facilitated by considering the failure of a variety of substances to inhibit or modify the phenomenon of local tissue reactivity.

In published(2) and unpublished experiments by one of the authors (G.S.), the substances employed were administered repeatedly by various routes. The substances which failed to suppress the phenomenon were acetylcholine, adenosine-5-phosphoric acid, alypin, antiplatelet serum, amino acids in various combinations, ascorbic acid, atropine, benadryl, biotin, calcium chloride, calcium gluconate, casein hydrolysate, choline, cocaine, congo red, curare, DCA, dicumarol, distilled water, estradiol, ether general anesthesia, folic acid, glucose, heparin, hesperidin, histaminase, histamine, india ink, inositol, lemon "citrin," milk, Niagara sky blue, nicotinic acid, pantothenic acid, paraminobenzoic acid, physostigmine hydrochloride, pilocarpine, progesterone, pyribenzamine, pyridin (in doses reducing significantly the blood platelet count), pyridoxal, pyridoxamine, pyridoxine, rat liver extract, rat spleen extract, riboflavin, sodium oxalate, sulfadiazine, sulfanilamide, sulfathiazole, thiamine,  $\alpha$ -tocopherol, trypan blue, trypsin, urethane, vitamin K (water soluble and oil soluble prepara-

tions), wheat germ oil, and yeast extract. Becker(3) showed that BAL, mapharsen, partial exsanguination and thyroidectomy failed to inhibit the phenomenon, while Smith and Humphrey observed no effect with antisan(4).

*Substances capable of suppressing the phenomenon of local tissue reactivity.* The bacterial preparations used for the preparatory and provocative injections were meningococcus, 44B, "agar washings" filtrates(2). Rabbits were prepared by a single intradermal injection of 0.25 ml of the filtrate diluted 1 : 2 and injected intravenously 24 hours later with 125 to 150 multiples of the minimal provocative dose. The doses employed gave large strongly positive reactions in over 90% of the control animals.

As may be seen from Table I, cortisone gave consistent inhibition of the phenomenon, when administered intramuscularly 2 hours prior to the provocative injection of the bacterial filtrate. Longer (Group 7) and shorter intervals (not recorded in the table) between the administration of cortisone and the provocative injection of the bacterial filtrate were less effective. The dose of cortisone required for consistent suppression was approximately 6 times greater than that of ACTH.

Lewin and Wassen(5) reported beneficial clinical effects similar to ACTH and cortisone from DCA and ascorbic acid. Thus we studied the effect of these substances on the phenomenon, (Group 1). The treatment failed to modify the phenomenon.

Smith and Humphrey(4) reported recently that sodium salicylate was capable of suppressing the phenomenon elicited with *E. coli*

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2. Shwartzman, G., *Phenomenon of Local Tissue Reactivity*. Paul B. Hoeber, Inc., New York, 1937.

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5. Lewin, E., and Wassen, E., *Lancet*, 1949, v2, 993.

TABLE I. Suppression of the Phenomenon of Local Tissue Reactivity.

Group No.	Substance	Treatment		Results		
		Total dose	Method of administration	Positive	Doubtful	Negative
1	DCA and Sodium ascorbate	10 mg 1 g	I.M. 2 hr prior prov. inj. I.V. 10 min. prior prov. inj.	5	1	0
2	Cortisone	12.5 mg	I.M. 2 hr prior prov. inj.	4	2	0
3	"	25 "	"	2	1	3
4	"	40 "	"	4	2	0
5	"	50 "	"	2	0	1
6	"	75 "	"	1	1	4
7	"	60 "	I.M. 4 hr prior prov. inj.	2	0	1
8	Sodium salicylate	1.6 g/kg	I.P. 20 min. prior prep. inj. 18 hr, ½ hr prior prov. inj. and 2 hr after prov. inj.	12	0	17
9	Calcium pantothenate	0.8 "	"	6	0	0
10	Sodium salicylate and Calcium pantothenate	1.6 " 0.8 "	"	0	1	5
11	Sodium salicylate and Calcium pantothenate	1.6 " 0.4 "	"	0	0	10
12	Sodium salicylate and Calcium pantothenate	1.6 " 0.2 "	"	2	0	4
13	Sodium salicylate and Cortisone	1.6 " 50 mg	I.M. 2 hr prior prov. inj.	1	0	5
14	ACTH	12.5 mg	I.M. 2 hr prior prov. inj.	2	0	14
15	Sodium salicylate and ACTH	1.6 g/kg 7.5 mg	I.P. 20 min. prior prep. inj. 18 hr, ½ hr prior prov. inj. and 2 hr after prov. inj. I.M. 2 hr prior prov. inj.	2	0	3
Controls for groups 1-15	—	—	—	65	3	3

Prep. inj. = Intradermal preparatory injection of the bacterial filtrate; prov. inj. = intravenous provocative injection of the bacterial filtrate; I.M. = intramuscularly; I.V. = intravenously; I.P. = intraperitoneally.

toxin. In some rabbits the reaction was completely inhibited, while in others the size of the reaction was markedly reduced. In our studies (Table I, Group 8) using a stronger toxin (*meningococcus*) the phenomenon was completely inhibited in 17 out of 29 rabbits, while in the remaining animals the reactions were strongly positive. The size of the reactions was not reduced.

Calcium pantothenate alone failed to produce any effect upon the phenomenon (Table I, Group 9). However, optimum combined doses of sodium salicylate and calcium pantothenate resulted in suppression of the phenomenon in all animals tested (Group II). Apparently, calcium pantothenate enhanced the suppressing effect of sodium salicylate without directly affecting the phenomenon.

Apparently the addition of sodium salicy-

late may enhance the effect of an insufficient dose of cortisone, while the action of ACTH in subminimal concentration is not improved by the addition of sodium salicylate (Groups 5, 13 and 15).

*Discussion.* The most important feature of the phenomenon of local tissue reactivity is profound vascular damage in the prepared site, which can only be elicited when the provocative injection is made into the general circulation. No satisfactory explanation is yet available concerning the necessity of introducing the provocative agent into the vascular system. Several possible mechanisms may be considered.

The possibility that a disturbance in the coagulation system is necessary for the elicitation of the reaction is not supported by the fact that agents capable of altering blood

coagulation, namely, distilled water, heparin, dicumarol, vitamin K, calcium salts, sodium oxalate, pyridine, and anti-platelet serum fail to produce any effect on the phenomenon. The role of the peripheral and central nervous system in the provocation of the phenomenon is excluded by lack of effect of general anesthesia and a variety of local anesthetics, atropine, acetylcholine, physostigmine, curare and pilocarpine. The suggestion that the reaction may be caused by release of histamine following the provocative injection is not borne out by the inability of histamine to provoke the reaction and the failure of histaminase and antihistaminics to exert any influence. Assumption receiving most support is that the damage to vascular endothelium necessary for the production of the reaction can be elicited only when the provocative agent is brought in contact with the endothelium intravascularly. Becker recently showed that  $\text{HN}_2$ , benzol and x-ray radiation(3) are capable of suppressing the phenomenon. He postulated that these agents produce the suppression by rendering the vascular endothelium anergic in the prepared site, thus preventing it from reacting to the provocative agent. Recently Schlang(6) confirmed the observations of Becker. In studying the mechanism of this inhibition he found that protection of the prepared site from the effect of  $\text{HN}_2$  failed to influence the inhibition of the reaction. In contrast, protection of the lower limbs from the action of  $\text{HN}_2$  by aortic occlusion decreased or prevented the inhibition. He concluded that the effect of  $\text{HN}_2$  was the result of systemic rather than local action.

Our observed suppression of the phenomenon by ACTH and cortisone indicates that provocation of the phenomenon depends on adrenal cortical function. Then the reported suppression of the phenomenon by sodium salicylate needs further examination. Drugs and certain environmental factors are capable of influencing adrenal cortical function, as judged by the decrease in concentration of adrenal ascorbic acid(7). Possibly then, the suppressing effect of sodium salicylate on

the phenomenon is indirect, being mediated by the adrenal cortex. Thus, we examined the effect of pantothenic acid. Pantothenic acid deficiency produces adrenal cortical damage(8) and probably some adrenal cortical dysfunction. According to Gaunt, Liling and Mushett(9), rats on a diet deficient in pantothenic acid show a decreased resistance to water intoxication. Sodium salicylate gave suppression of the phenomenon only in 17 out of 29 rabbits tested. Since the effect of sodium salicylate may be mediated through the adrenal cortex it seemed of interest to determine the effect of pantothenic acid on the action of sodium salicylate. Pantothenic acid enhanced the suppressive action of sodium salicylate on the phenomenon, since pantothenic acid in itself fails to show any effect.

Although non-anaphylactic in nature, the phenomenon is due to a profound alteration in vascular reactivity. Direct and indirect clinical evidence strongly suggests that the phenomenon demonstrates the mechanism underlying the production of a variety of spontaneous diseases and syndromes of known and unknown etiology in which vascular lesions are a predominant feature(1,10). These diseases fall into the spectrum of therapeutic activity of ACTH and cortisone. The ability of the agents to suppress the phenomenon points again to the role the phenomenon plays in the elicitation of these diseases and at the same time offers an experimental approach for the elucidation of the beneficial therapeutic effect of these

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9. Gaunt, R., Liling, M., and Mushett, C. W., *Endocrinology*, 1946, v38, 127.

10. Black-Schaffer, B., *Arch. Path.*, 1947, 43, 28; Bordet, J., *Traité de l'immunité dans les maladies infectieuses*. Masson et Co., Paris, 1939; Shwartzman, G., Klemperer, P., and Gerber, I. E., *J. Am. Med. Assn.*, 1936, v107, 1946; Shwartzman, G., and Gerber, I. E., *Ann. N. Y. Acad. Sci.*, 1948, v49, 627.

agents. Since the combined treatment with sodium salicylate and calcium pantothenate proved effective experimentally, the clinical trial of the combination may be worthy of consideration.

*Summary.* ACTH, cortisone and sodium salicylate were capable of suppressing the phenomenon of local tissue reactivity. The dose of cortisone required for the inhibition was approximately 6 times greater than that of ACTH. Sodium salicylate completely in-

hibited the phenomenon in 17 out of 29 rabbits while in the remaining animals the reactions were strongly positive. Pantothenic acid, which had no effect upon the phenomenon by itself, enhanced significantly the suppressing effect of sodium salicylate.

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### Demonstration of Increased Gonadotrophic Hormone Production in Castrated Mice with Intrasplenic Ovarian Grafts.\* (18137)

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Granulosa cell tumors, luteomas and mixed tumors occur in intrasplenic or intrapancreatic ovarian grafts in castrated rats(1) and mice(2). It has been demonstrated that the livers of animals of these species can inactivate estrogenic hormones(3-5) and that castrated rats or mice with intrasplenic ovarian grafts remain in diestrus after the first few weeks from the time of grafting(1,2). Therefore, it has been concluded that the pituitary glands of these animals are of the castrate type and produce greatly increased amounts of gonadotrophic hormones, with the resultant formation of ovarian tumors. In support of this concept, it has been shown (1,2,6) that the presence of an intact ovary,

or of vascular adhesions between the graft and the body wall or the uterus, or the administration of estrogenic or androgenic hormones prevents the occurrence of ovarian tumors in intrasplenic ovarian grafts. No direct determination of the rate of production of gonadotrophic hormones in castrated animals with intrasplenic ovarian grafts has been reported. However, Jungck, Heller and Nelson(7) bioassayed the pituitary glands of castrated rats with intrasplenic ovarian grafts and found only a slight increase in gonadotrophic potency, with an equal increase in the rats with adhesions between the graft and the body wall. Their experiment was not critical, since the gonadotrophic content of the pituitary gland may not be proportional to the rate of release of gonadotrophins from it(8). The technic of parabiosis provides a method for assaying circulating gonadotrophins. The output of pituitary gonadotrophins is increased in castrated(9) and x-rayed(10) rats as de-

\* This investigation was aided by grants from The Anna Fuller Fund, the National Cancer Institute (U.S.P.H.S.), and the James Hudson Brown Fund of Yale University School of Medicine.

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TABLE I. Ovarian, Uterine and Body Weights of Parabiotic Mice, One Member Castrated and Bearing an Intrasplenic Ovarian Graft.

Group	Animal	No.	Days in parabiosis	Body wt (g)	Ovarian wt (mg)	% increase	t	P	Uterine wt		% increase	
									Normal (mg)	Grafted (mg)	Normal	Grafted
1*	11	20	36.0	6.8					40.0	36.2		
	12	12	34.3	6.6					31.6	34.6		
	13	11	34.5	7.8					38.0	32.6		
	16	35	40.7	10.6					48.2	31.4		
	17	15	37.1	5.8					38.4	34.0		
	18	15	32.0	8.0					43.4	34.0		
	22	13	30.2	8.8					52.4	34.4		
Avg		17	35.0	7.8	4	0.4	0.70		41.7	33.9	-30	-3
2†	27	11	35.6	6.6					32.8	34.6		
	28	9	40.8	13.8					114.0	31.0		
	31	12	36.2	8.2					40.6	34.6		
	33	13	38.8	13.4					52.6	24.4		
	34	12	38.5	10.6					125.0	36.6		
	35	12	39.9	11.4					62.4	25.6		
	38	10	37.6	11.2					86.0	25.8		
Avg		11	38.2	10.7	43	2.5	0.04		73.3	30.4	23	8
3‡	25	5	32.5	5.6					64.6	29.2		
	29	4	33.7	7.6					53.4	20.2		
	30	4	35.3	8.2					52.0	24.2		
	32	2	35.2	8.8					73.2	45.2		
	36	7	36.8	7.4					54.6	46.4		
	Avg	4	34.7	7.5					59.6	33.0		

\* Castration, intrasplenic transplantation and parabiosis performed at the same time.

† Castration and intrasplenic transplantation carried out 30 to 50 days prior to parabiosis.

‡ Same as Group 2, but autopsied from 2 to 7 days after parabiosis; served as controls.

terminated by this technic. In the present experiment, parabiotic mice were used to demonstrate an increased gonadotrophic hormone output by the pituitary glands of castrated mice with intrasplenic ovarian grafts.

*Material and methods.* Forty female mice of the Strong A strain were castrated at one to 2 months of age, and in each an ovary was transplanted into the spleen as an autologous or homologous graft. Each mouse was placed in parabiosis with a female litter mate; the operation was performed at the same time as grafting in 22 of the mice and 30 to 50 days later in 18 of the mice. The operation was performed as follows: A large segment of skin, extending from the base of the ear to the base of the tail, was removed from the right side of one and the left side of the other member of the pair. An incision was made through the abdominal musculature of each mouse, and the 4 cut muscle edges were sutured with a continuous silk suture. Anchoring sutures were placed through

the adjacent scapulae and pelvic muscles. The skin edges were then approximated and held by a continuous silk suture. The parabiotic mice were kept in small cages in groups of from one to three pairs. Purina fox chow and water were available at all times. Some of the mice that received ovarian grafts from 30 to 50 days before being placed in parabiosis were killed or died from 2 to 7 days after parabiosis (Group 3) and served as controls. The remainder (Group 2), and the mice which received grafts at the same time parabiosis was performed (Group 1), died or were killed from 9 to 35 days after parabiosis. Twelve of the pairs pulled apart partially or completely; in 4 others there were vascular adhesions between the graft and the body wall. Mice with either of these conditions were discarded. Another 5 pairs were not autopsied because of advanced post-mortem changes. The total body, ovarian and uterine weights of the remaining 19 pairs were determined at autopsy.

*Results.* It will be seen from Table I, which summarizes the results of these experi-

ments, that the average duration of parabiosis for the mice in which castration, intrasplenic transplantation and parabiosis were performed at one time (Group 1) was 17 days, with a range of from 11 to 35 days. The mice which were castrated and intrasplenic transplantation performed 30 to 50 days prior to parabiosis were divided into 2 groups in which the average duration of parabiosis was 11 days (Group 2) and 4 days (Group 3), with ranges of from 9 to 13 days and from 2 to 7 days, respectively. The body weights of the mouse pairs averaged 35.0, 38.2 and 34.7 g in the 3 groups. The average ovarian weight of the intact parabionts of the pairs in which castration and intrasplenic transplantation were carried out 30 to 50 days prior to parabiosis (Group 2) showed an increase of 43% over the controls (Group 3), while the pairs in which all operative procedures were performed at the same time (Group 1) showed only a 4% greater ovarian weight than the controls (Group 3). A comparison of the ovaries of the 2 groups (1 and 2) shows that castration and the presence of the graft for 30 to 50 days longer in the parabiont produces a 37% greater increase in the size of the ovaries. The same trend is evident in the pairs with the longest period of parabiosis in Group 1 (Table I); the ovarian weight of the mouse surviving in parabiosis for 35 days was 10.8 mg, in contrast to the 7.8 mg average for the group.

The average uterine weight of the intact parabionts in group 2 was 23% greater than in Group 3 which served as controls, and 73% greater than in Group 1 where all of the operations were performed at one time. The data for Group 1 is a measure of the very early changes. The average uterine weight of the castrated-grafted parabionts reflected the absence of estrogen stimulation and a certain amount of regression which was proportional to the length of time after castration and grafting. Thus, in mice in which castration and intrasplenic transplantation were done 30 to 50 days before parabiosis (Group 2) the average uterine weight was 8% less than in the controls (Group 3) and

UTERINE AND OVARIAN RESPONSES OF INTACT PARABIONTS  
OF CASTRATED MICE BEARING INTRASPLENIC OVARIAN GRAFTS

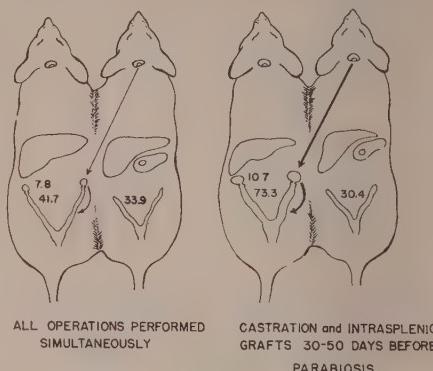


FIG. 1.  
A schematic representation of the endocrine situation under the two conditions of parabiosis (Group 1 and 2). In each case the animal on the right is castrated and bears an ovarian graft in the spleen.

11% less than in Group 1 where all operations were performed at the same time. Fig. 1 shows a schematic comparison of Groups 1 and 2.

**Discussion.** The 43% increase in ovarian weight of the mice placed in parabiosis with animals that had been castrated and had received ovarian grafts in the spleen 30 to 50 days previously, and were autopsied 9 to 13 days later, is indicative of an increased rate of production of gonadotrophins by castrated mice with intrasplenic ovarian grafts. The lack of any significant increase in ovarian weight (4%), even during a slightly longer period of parabiosis in the pairs in which all the operative procedures were performed at the same time (Table I) does not conflict with this interpretation but rather indicates that there is no measurable increase in production of gonadotrophins during the first weeks after castration and grafting of an ovary into the spleen. The observations of Evans and Simpson(11) who noted a slow increase in pituitary gonadotrophic potency after castration in the rat might be taken to indicate that this increase could not be picked up by parabiotic experiments during the first

11. Evans, H. M., and Simpson, M. E., *Am. J. Physiol.*, 1929, v89, 371.

two weeks after castration. However, Biddulph, Meyer and Gumbreck(12) have shown that following simultaneous parabiosis and castration of one member of the pair the increased production of gonadotrophic hormone is easily measurable by the increased weight of the ovaries of the intact parabiont before the 11th day of parabiosis. If the mouse behaves the same as the rat, this would suggest that the increase in gonadotrophins in the castrate is somewhat inhibited by the presence of the ovarian graft in the spleen. With the increased stimulation of the ovaries of the intact member and the absence of the transfer of estrogen to the other member of a pair(13), the uterine responses are what would be expected.

The effects measured during the first two weeks of parabiosis may not be completely free from the influence of factors inherent in the operative procedure, although the findings of Biddulph, Meyer and Gumbreck(12) showed that fewer than 13% of the ovaries of intact female rats in parabiosis with castrates (both operations performed simulta-

eously) failed to hypertrophy by the 11th day of parabiosis. However, the absence of any significant ovarian hypertrophy in mice of Group 1, despite an average duration of 17 days in parabiosis, renders the 43% increase in ovarian weight that occurred in Group 2 in an average time of 11 days highly significant. It is realized that these experiments measure only the beginning of the gonad-hypophyseal imbalance present in a castrate bearing an ovarian graft in the spleen and that long term experiments in parabiosis are essential for its full elucidation.

**Summary.** Evidence of an increased rate of production of gonadotrophins in castrated mice with intrasplenic ovarian grafts has been obtained by means of parabiotic experiments. The ovaries of normal mice placed in parabiosis with female littermates that had been castrated and had an ovary grafted into the spleen from 30 to 50 days previously averaged 37% heavier than the corresponding ovaries when parabiosis was performed simultaneously with the other operations. The duration of parabiosis ranged from 9 to 13 and from 11 to 35 days, in the 2 groups, averaging 11 days in the former and 17 days in the latter.

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### Inhibitory Effect of Cortisone on Anaphylaxis in the Mouse.\* (18138)

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Preliminary studies of electrolyte changes in the plasma and tissues of mice during anaphylaxis indicated that profound primary disturbances in the intracellular components of sensitized tissues follow the rapid union of antigen and antibody and that these

changes, in turn, may cause the secondary hypovolemia and the shock-like circulatory collapse which follow(1).

The nature of these chemical alterations suggested to us that they might be inhibited, at least in part, by gluco-corticoid compounds. Should this be true, it would be of considerable importance. The question is of further interest inasmuch as it is now known that

\* This investigation was supported in part by grants from Ciba Pharmaceutical Products, Inc., Summit, N. J. and The Division of Research Grants and Fellowships, National Institutes of Health, Bethesda, Md.

1. Fox, C. L., Jr. and Nelson, C. T., unpublished data.

neither ACTH nor Cortisone (17-hydroxy-11-dehydrocorticosterone) can influence the course of anaphylactic shock in the histamine-susceptible guinea pig(2,3). The following investigation was undertaken in order to study the effect of Cortisone on anaphylactic shock in the mouse which, in contrast to the guinea pig, is relatively resistant to injected doses of histamine.

*Methods.* Male and female Swiss mice weighing between 20-30 g were sensitized by intraperitoneal injections of 1 cc of undiluted normal horse serum every second day for a total of 4 cc. This is the method previously employed by Mayer and Brousseau(4). Eleven to 15 days after the last sensitizing injection each of these animals received 1 cc (or 4% of the body weight—whichever was less) of the same antigen by rapid injection into a tail vein.<sup>†</sup> The animals were divided at random into two groups: Group I consisting of 34 control mice, and Group II comprising 42 Cortisone-treated animals. The mice in Group II received a single intramuscular injection of Cortisone (Merck) into the thigh 18 hours prior to the administration of the shocking dose of antigen. The amounts of Cortisone employed ranged from 0.75 mg to 3.0 mg per animal. After injection of the challenging dose of antigen the animals were observed closely for the appearance of the signs of anaphylaxis. Anaphylactic shock in the mice was characterized initially by sluggishness, mild dyspnea, slight cyanosis, and occasionally, scratching of the nose. If the signs did not progress beyond this point the anaphylactic shock was graded as mild. As the shock deepened, the mice exhibited severe dyspnea, marked cyanosis, wobbling gait, loss of sphincter control, and ultimately, weakness of the extremities, convulsions, cycling movements of the legs and death. In animals showing this degree of anaphylaxis,

TABLE I. Inhibitory Effect of Cortisone on Anaphylactic Shock in the Mouse.

	Cortisone, mg	No. sensitized	Symptoms of anaphylactic shock				% showing fatal shock
			None	Mild	Severe	Fatal	
Sensitized controls	0	34	0	0	6	28	82
Sensitized cortisone-treated	3	22	12	10	0	0	0
	1.5	10	4	5	0	1	10
	0.75	10	0	4	3	3	30

the shock was graded as severe if they did not die, and fatal if death occurred. The signs of anaphylaxis did not appear in less than 4 minutes and did not terminate in death in less than 10 minutes, or more than 49 minutes.

*Results.* The results of these experiments are summarized in Table I. The control animals in Group I without exception showed signs of severe anaphylactic shock and in 28 of 34 animals (82%) the shock was fatal. On the other hand, in Group II, the Cortisone-treated group, none of the 22 animals receiving 3.0 mg of Cortisone exhibited severe or fatal anaphylactic shock. No more than mild symptoms of anaphylaxis were observed in any of these animals and, indeed, in 12 of them not even mild shock supervened. Mice which received smaller amounts of Cortisone (*i.e.* 0.75 mg or 1.5 mg) also showed a high degree of protection from severe anaphylaxis. In these animals, however, protection was not complete and in 4 of 20, fatal anaphylactic shock occurred. These results indicate that Cortisone, administered in the dosage and under the conditions of this experiment, has an inhibitory effect on anaphylactic shock in mice.

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4. Mayer, R. L. and Brousseau, D., *Proc. Soc. EXP. BIOL. AND MED.*, 1946, v63, 187.

<sup>†</sup> This amount of undiluted normal horse serum given intravenously produced no toxic manifestations in 32 normal (unsensitized) mice.

As might be expected, the administration of a single dose of Cortisone afforded only temporary protection against anaphylactic shock. Mice in the Cortisone-treated group which survived the challenging dose of antigen were given another shocking dose of undiluted normal horse serum intravenously 11 days later. In all instances the animals

showed signs of anaphylactic shock and the incidence of fatalities (78%) did not differ significantly from that observed in the original sensitized control group (Group I).

The duration of the inhibitory effect of a single intramuscular injection of Cortisone was tested in additional groups of sensitized mice. Complete protection from fatal anaphylaxis was observed as early as 6 hours after the administration of 3.0 mg of Cortisone and this persisted for at least 48 hours. After 96 hours, however, the protective effect had largely been dissipated since at this point the challenging dose of antigen produced fatal anaphylactic shock in approximately 40% of the animals.

**Summary.** The administration of Cortisone to 42 sensitized mice 18 hours before the intravenous injection of a challenging dose of antigen prevented fatal anaphylactic shock in 38 animals. In contrast, 28 of 34 sensitized but unprotected control mice died in anaphylactic shock.

The authors wish to thank Mr. Bernard K. Friedman and Mrs. Jacqueline Isola for their technical assistance in this work.

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### Effect of Testosterone Propionate on Total Urinary Nitrogen Excretion of the Rat Following Burns.\* (18139)

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Trauma is frequently followed by an increased total urinary nitrogen excretion (1-4). It is the consensus that this increased excretion of nitrogen represents increased tissue breakdown or failure of tissue formation with subsequent signs and symptoms of protein depletion. A substance which would promote tissue synthesis might be of value to prevent or ameliorate the accompaniments of protein depletion in damaged individuals. Testosterone and related compounds are substances which have been shown to be capable of lowering urinary nitrogen excretion (5-7) and

causing muscle tissue and organ hypertrophy with concomitant increase in total protein and water content of these tissues (8-10).

In view of these facts it was considered important to determine the effect of testosterone propionate on the urinary nitrogen excretion following burns. It was also hoped that such a study might aid in the elucidation of the mechanism of the metabolic re-

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\* The opinions expressed in this paper are those of the authors and do not necessarily represent the official views of any governmental agency.

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4. Brunschwig, A., Clark, D. E., and Corbin, N., *Ann. Surg.*, 1942, v115, 1091.

TABLE I. Composition of Liquid Diet.

Sucrose	860 g
Egg albumin (Merck)	160 "
Cellu flour (soy bean)	120 "
Dried brewers' yeast powder	100 "
Salt mixture*	40 "
Cod liver oil	30 "
Mazola oil	30 "
Wheat germ oil	30 "
Water to make	2000 ml

\* Salt mixture: Primary sodium phosphate, 14.0 g; calcium chloride, 12.0 g; monobasic potassium phosphate, 5.2 g; cupric sulfate, 4.0 g; ferric citrate, 3.0 g; magnesium sulfate, 1.0 g; manganese sulfate, 0.8 g; zinc chloride, 10 mg; potassium iodide, 10 mg.

sponse to damage. Previous work on this problem has been inconclusive. The clinical investigations(11-15) were of necessity inadequately controlled and in one unsatisfactory study with animals(16) only 2 dogs were used.

**Methods.** Adult female and young adult male albino rats of Harlan stock were adapted for 6 days to the force-feeding(17) of a liquid diet (Table I) modified from Ingle *et al.*(18). From the end of the adaptation period to the end of the experiment each of the animals was given 11 ml of this diet 2 times daily. By calculation the diet yielded about 0.2 calorie per g of body weight per

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day and was made up, on a caloric basis, of 18% protein, 63% carbohydrate, and 19% fat. During the experimental period the animals were kept individually in metabolism cages from which the urine was collected every 2 days. Before collection it was preserved with dilute sulfuric or oxalic acid and toluol, and after collection it was kept in a refrigerator or deep freeze until the total nitrogen determinations were carried out by the macro- or micro-Kjeldahl procedure.

Five groups of animals were studied, of which 3 were burned. After a suitable control period, the first group, female animals, was given daily subcutaneously either 2.0 mg of testosterone propionate in 0.2 ml of peanut oil (7 animals) or just 0.2 ml of peanut oil (5 animals). Two days after the onset of this medication they were burned. The second and third groups, male animals, varied only in the time sequence of the start of the medication with respect to the burn. In one, the medication was started 12 hours before the burn (7 and 5 animals) and in the other, 4 days after the burn (7 and 6 animals). The fourth group consisted of females which were just given 2.0 mg of testosterone propionate in 0.2 ml of peanut oil (6 animals), and the fifth group consisted of males which were given similar doses of testosterone propionate in peanut oil (5 animals) or just peanut oil (5 animals). Under intraperitoneal pentobarbital anesthesia in Groups 1 and 2 and ether anesthesia in Group 3, an approximately 30% area burn was produced by dipping the clipped backs of the rats into a constant temperature water bath at 73°C for 30 seconds after the method of McCarthy (19). The total urinary nitrogen excretion was followed after the burn procedure for a length of time sufficient for the effectiveness of the testosterone propionate to become statistically significant and then disappear. A period of 10 to 12 days usually sufficed. In the case of the animals not burned but just given testosterone propionate in peanut oil or peanut oil alone, the urinary nitrogen excretion was followed after the on-

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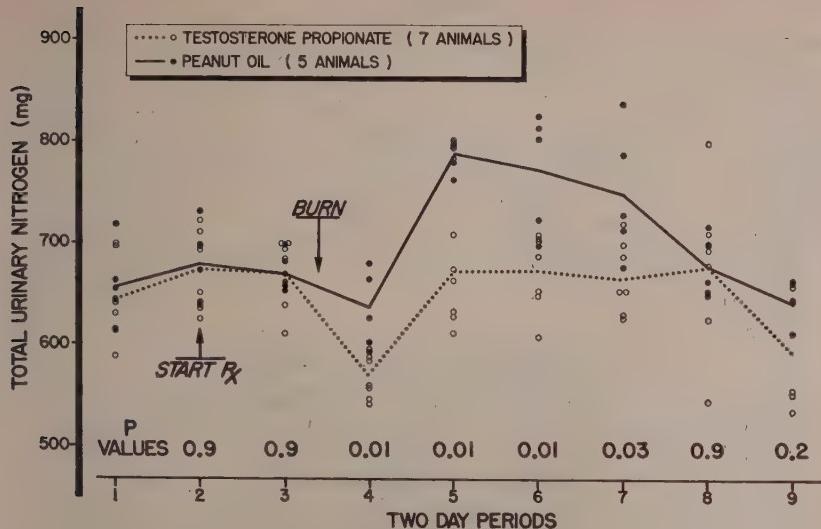


FIG. 1.  
Effect of thermal injury and subcutaneous testosterone propionate and peanut oil administration on urinary nitrogen excretion of adult female rats.

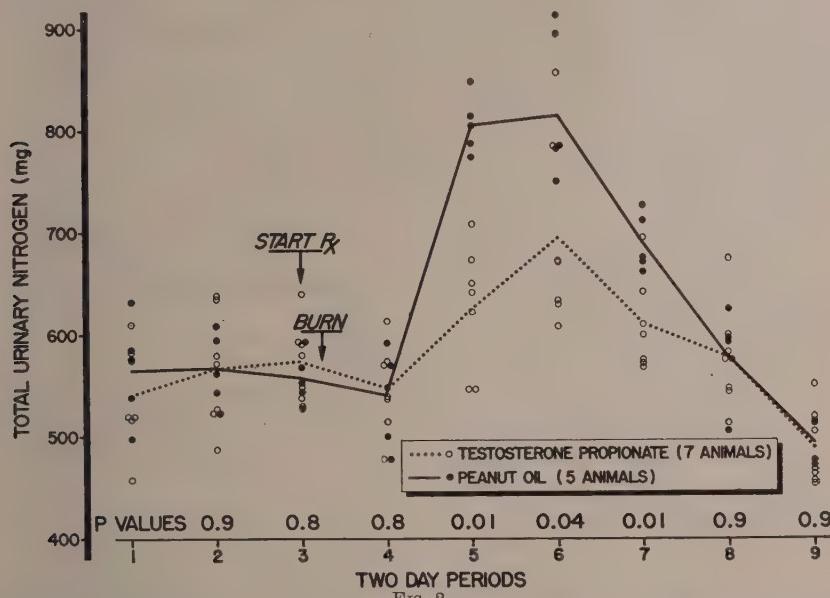
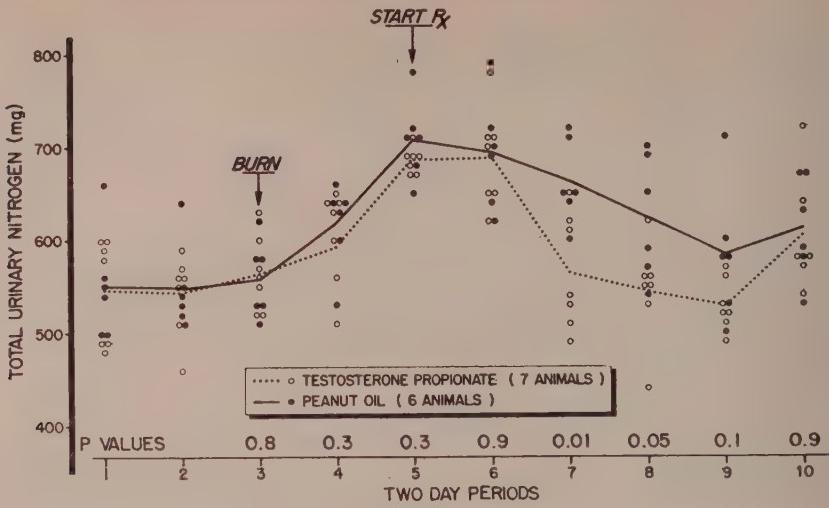


FIG. 2.  
Urinary nitrogen excretion following thermal burns of young adult male rats. Effect of subcutaneous testosterone propionate and peanut oil administration started 12 hours before the burn.

set of the medication until it returned to the control level, spontaneously in the case of the males (14 days), or after stopping the

medication in the case of the females 18 days after its inception.

*Results.* The burn procedure used re-



Urinary nitrogen excretion following thermal burns of young adult male rats. Effect of subcutaneous testosterone propionate and peanut oil administration started 4 days after the burn.

sulted in a fairly uniform third degree burn of approximately a third of the total body surface area as judged by histologic examination<sup>†</sup> and an estimate of the total area of skin showing changes characteristic of a burn of this degree. At about the eighth post-burn day the involved skin appeared as a thick, dark crust without gross evidence of infection.

Fig. 1, 2, and 3 depict the data obtained on the urinary nitrogen excretion of burned animals. In the control peanut oil group there was an increase in total urinary nitrogen excretion beginning approximately the second to fourth post-burn day and ending approximately on the tenth to twelfth post-burn day in both sexes. When the nitrogen excretion of those animals which were burned but given testosterone propionate is compared with the foregoing, it can be seen that the rise expected after burning is markedly depressed by the testosterone propionate administration in both sexes beginning two to three days after the onset of the medication.

Fig. 4 and 5 show that the administration of 2.0 mg of testosterone propionate daily to normal male and female animals resulted in a maximal fall in urinary nitrogen excretion of approximately 140 mg of nitrogen per two-

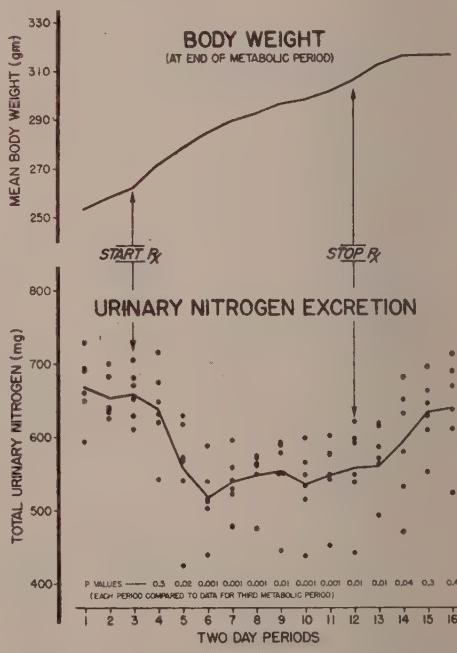


FIG. 4.

Effect of subcutaneous testosterone propionate administration on body weights and urinary nitrogen excretion of 6 adult female rats.

day period in the female and approximately 100 mg of nitrogen per two-day period in

<sup>†</sup> Kindly performed by Dr. C. L. Pirani.

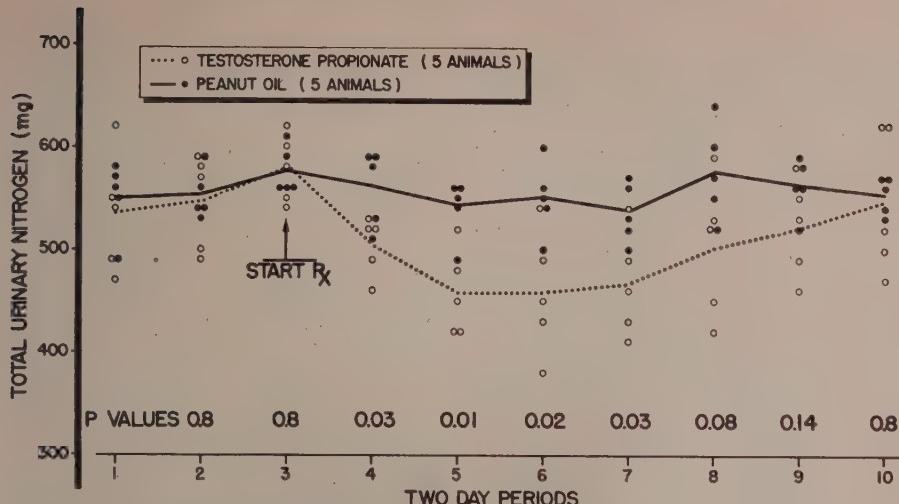


FIG. 5.

Effect of subcutaneous testosterone propionate and peanut oil administration on urinary nitrogen excretion of young adult male rats.

the male animals. The depression began in both sexes two to four days after the onset of the medication. It continued for from 10 to 14 days in the case of the males after which time it apparently spontaneously disappeared. The depression in the urinary nitrogen excretion of the females continued for as long a time as the drug was given (18 days). After this time, the drug was discontinued and the nitrogen excretion rose toward the pre-injection levels.

On comparing the calculated effect of testosterone propionate in the burned animals with its effect in the normal animals, in both sexes it appears to be essentially unchanged by the burn for about 8 days, but thereafter disappears. In the unburned males the effect possibly lasts longer than in the burned, but in the females it certainly lasts longer in the unburned than burned.

The results of a statistical analysis using Fisher's "t" test of significance of the aforementioned changes are shown in Fig. 1-5. According to these calculations the changes described are significant.

**Discussion.** It has been shown in this study that for a certain time interval the effect of testosterone propionate on the urinary nitrogen excretion of the burned rat is

essentially equivalent to its effect in the normal animal. If the albino rat and the human being are sufficiently similar as to their metabolic reaction to thermal trauma and testosterone propionate administration, one might predict that for a certain period post-burn, the administration of testosterone propionate would save the individual approximately 0.03 to 0.1 g of nitrogen per kg per day(5,20), or in the case of a 70 kg subject, from 57 to 200 g of tissue per day(21).

Whether this result would be of benefit is questionable in view of some possible side effects of the drug. These are the enhancement of signs of masculinization following burns(22), a possible adverse effect on healing at the burn site, and an increase in the retention of water and salts following trauma (5,23).

Following thermal injury(22) and other types of damage(15) the 17-ketosteroid ex-

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cretion of female and male patients is at first briefly increased, then markedly decreased. Two explanations of this decrease in 17-ketosteroid excretion are more acceptable than others. They are that either there is a decreased production and secretion of the precursors of these compounds or that these precursors are metabolized differently following damage than in the normal organism. It has been demonstrated that in eunuchoid humans(5) and castrate albino rats(7) with a subnormal production of testosterone-like compounds, the effect of administered testosterone on nitrogen excretion is increased over that found in the normal organism. Therefore, if there was a decreased secretion of the 17-ketosteroid precursors following damage, one might expect the quantitative action of testosterone propionate to be increased in the burned organism over the normal response. It has been shown in this study that the effectiveness of testosterone propionate on the urinary nitrogen excretion is markedly shortened in female and possibly shortened in male animals following burns. There are no data available as to the quantitative changes of the 17-ketosteroid excretion of rats following burns. If the pattern is similar to that described in humans, the findings in this study suggest the second of the two postulates, that the testosterone compounds are metabolized differently following damage. This possibility is further borne out by the findings of Forsham *et al.*(24) and Mason *et al.*(25). These workers found increased excretions of 17-ketosteroids and 11-oxysteroids following the injection of purified ACTH into humans. That the hypophyseal, adrenal cortex mechanism is activated following burns has been demonstrated by Harkins and Long(26) and Venning *et al.*(27). Therefore, that there is

an increased production of 17-ketosteroid precursors following burns seems possible.

It can be seen in Fig. 1 and 2 that in the case of the burned females and the first group of burned males the disappearance of effectiveness of testosterone propionate coincides with the return of the nitrogen excretion of the control animals to the pre-burn level. Whether this temporal relationship is causal or coincidental is unknown. It is interesting to note that in the case of the second group of burned males (Fig. 3) in which the medication was begun four days after the burn, the response to testosterone is similar to that in the first group of male animals in which the testosterone was started twelve hours before the burn. We have tried to extend this investigation to include observations on the effectiveness of testosterone propionate when started 10 to 20 days after the burn. These attempts have been seriously handicapped by several factors. These include a high mortality among animals force-fed at this time, an extreme variability in nitrogen excretion, and a tendency for a secondary rise in nitrogen excretion to occur beginning approximately 14 days post-burn.

**Summary.** The subcutaneous administration of testosterone propionate depressed the rise in urinary nitrogen excretion following burns in male and female rats. The effect of testosterone propionate in reducing the total urinary nitrogen excretion of male and female adult rats is essentially unchanged by a thermal burn for a period of 8 days after the burn or onset of the testosterone medication. For 2 to 4 days after this period the effect of the drug post-burn is no longer evident. In normal female animals the effectiveness of testosterone propionate continues for at least 18 days whereas in normal males it disappears at 12 to 14 days. Possible reasons for this wearing-off of the effectiveness of testosterone propionate after burns are considered. Various problems connected with the possible use of testosterone

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propionate in the damaged patient are discussed.

A part of this report is based on work done by Capt. Braasch in the Department of Physiology of the University of Illinois School of Medicine in

partial fulfillment of the requirements for the degree of Master of Science in Physiology. The authors wish to thank the Schering Corporation for a generous supply of testosterone propionate.

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### Blood Amino Acid Level and Adrenal Cholesterol Content During "Tourniquet Shock" in the Rat. (18140)

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It has been established that "shock" will produce an elevation of the blood amino acid level and a decrease in adrenal cholesterol content(1,2). The "sparing" effect of administration of isotonic sodium chloride solution in lowering mortality in traumatic shock has been recorded by various investigators (3-5). It was of interest to determine the effects of saline therapy on the blood amino acid level and the adrenal cholesterol content of rats in "tourniquet shock." The findings of this investigation are presented in this paper.

**Experimental.** White male albino rats of the Sprague-Dawley strain, weighing 220 to 290 g and fed on Purina dog chow checkers, were used. The procedure for shock production in mice developed by Rosenthal(4) and used by Harkins(5) for rats, was employed (6). The animals were fasted for 16 hours before the experiment but were permitted water. With the animals under light ether anesthesia, elastic rubber band (Eberhard

Faber No. 30) tourniquets were applied to the limbs and the teeth were clipped to prevent tourniquet removal or self-laceration. The animals were placed in individual cages without access to food or water. Depending on the type of experiment, the animals were sacrificed by decapitation without tourniquet release or tourniquets were removed and the animals replaced in the individual cages to be sacrificed at varying periods of time following release. For the experiments reported here, the occlusion was of both hind-limbs for 4½ hours and of hind- and fore-limb unilaterally for 3 hours or for 5 hours. For treatment of the induced "shock," a 0.9% NaCl solution was given intraperitoneally, 10 ml immediately following tourniquet release, 10 ml 2 hours later and 3½ hours after release the amount necessary to bring the total administered to 10% of the body weight. All experiments were conducted at room temperatures of 20 to 25°C. After decapitation of the animal, the adrenal glands were immediately dissected, weighed and analyzed for cholesterol according to the method of Schoenheimer and Sperry(7). The total cholesterol was determined in each case and the amount of cholesterol is reported as mg/100 mg of fresh wet adrenal tissue. Blood amino acid(8), urea(9), and hematocrit determinations were carried out on blood ob-

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TABLE I. Blood Amino Acid and Urea Levels and Hematocrits at Different Time Intervals After Release of Tourniquets Applied on Both Hind Legs for 4.5 Hr. Average values  $\pm$  standard deviation for No. of animals indicated in parentheses.

Time after release	Amino acid nitrogen (mg %)	Urea nitrogen (mg %)	Hematocrit, %
(Normal values)	12.3 $\pm$ 0.66 (14)	15.0*	45.7 (5)
0	10.8 $\pm$ 2.59 (18)	14.6 $\pm$ 2.91 (12)	45.8 (17)
30 min.	—	—	59.9 (11)
1 hr	13.75 $\pm$ 1.38 (13)	20.8 $\pm$ 3.89 (10)	61.1 (8)
2 "	16.15 $\pm$ 2.15 (11)	27.9 $\pm$ 2.65 (3)	61.3 (3)
4 "	18.0 $\pm$ 0.77 (4)	39.1 $\pm$ 0.22 (2)	—
9 "	20.0 $\pm$ 2.03 (11)	55.0 $\pm$ 11.51 (3)	—
16-17 "	20.7 $\pm$ 1.71 (12)	98.4 $\pm$ 41.70 (7)	67.0 (5)

\* Taken from Fraenkel-Conrat, J., Fraenkel-Conrat, H., and Evans, H. M., *Am. J. Physiol.*, 1942, v137, 200.

tained at the time the animals were killed by decapitation.

*Results and discussion.* By the method described, it was found that occlusion of both hind-limbs for 4½ hours or hind- and fore-limb unilaterally for 3 or 5 hours produced a shock-like state in all animals after the tourniquets were removed. These time periods yielded a satisfactory 24-hour mortality percentage for the purpose of this study. With tourniquets on both hind-limbs for 4½ hours, mortality 24 hours after tourniquet release for the untreated group averaged 89.5% compared with 9.76% average mortality rate for the saline treated animals(6). Occlusion of hind- and fore-limb unilaterally for 3 hours resulted in the death, in the 16 hours following tourniquet release, of 70% of the non-treated animals compared with an 18% average mortality rate in the saline treated animals. Tourniquets on hind- and fore-limb unilaterally for 5 hours produced an average mortality, 16 hours after tourniquet release, of 84% in the non-treated group and 10% in the saline treated group. The mortality figures, obtained both for treated and untreated animals, agree well with those recorded in the literature(3-6). The stress, produced by either occlusion of both hind-limbs for 4½ hours or hind- and fore-limb unilaterally for 5 hours, seems to be approximately of the same magnitude.

The data presented in Table I show that after tourniquet release at different time intervals, an early and progressive elevation in the

blood amino acid and urea nitrogen concentration occurs. The values for blood amino acid and urea nitrogen, obtained at the time of tourniquet release, (zero time), agree with those for normal untreated animals. From these results and those of experiments 4 and 8 (Table II), it is evident that the application of tourniquets *per se* for the time periods employed does not produce any elevation in these blood constituents. Blood amino acid nitrogen determinations on 24 animals sacrificed at varying intervals up to 22 hours without tourniquet release showed no changes from the normal control values. Apparently only after the release of the tourniquets can this change in blood amino acid and urea nitrogen concentration be observed (Table I and Exp. 5, 6, 9 and 11 of Table II).

Apparently, occlusion of the limbs for a period of several hours is, itself, a sufficient stress producing procedure to cause a lowering of the adrenal cholesterol content (Exp. 4 and 8 of Table II). On release of the tourniquets, there is a further progressive reduction in the adrenal cholesterol level (Exp. 5, 6, 10 and 11 of Table II). The finding that occlusion of the limbs *per se* produces no change in the blood amino acid and urea nitrogen concentration in spite of a definite increased activity of the adrenal cortex may perhaps be explained as follows: during the period of limb occlusion the organism is still capable of managing properly the accelerated formation of protein breakdown products brought about by the increased activity of the adrenal cortex. However, release of the tourniquets leads to an impairment in the

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TABLE II. Effect of Saline Administration on Blood Amino Acid Level and Adrenal Cholesterol Content During Tourniquet Shock. Average values  $\pm$  standard deviation for No. of animals indicated in parentheses.

No. of exp.	Manipulation and therapy	Blood amino acid nitrogen, mg %	Cholesterol content of fresh adrenal tissue, mg %
A. Controls—no tourniquets.			
1	None	12.0 $\pm$ .40 (9)	4.33 $\pm$ .89 (9)
2	Saline treatment	11.2 $\pm$ .77 (6)	4.75 $\pm$ 1.70 (9)
3	Anesthetized, teeth clipped, sacrificed 19 hr later	11.6 $\pm$ .62 (4)	4.75 $\pm$ .74 (4)
B. Tourniquets on hind- and fore-limb unilaterally for 3 hr, anesthetized and teeth clipped.			
4	Sacrificed at 3 hr, no t.r.*	12.5 $\pm$ .63 (10)	2.79 $\pm$ .46 (9)
5	Sacrificed 2 hr after t.r.	15.1 $\pm$ 1.01 (9)	1.61 $\pm$ .42 (10)
6	Sacrificed 16 hr after t.r.	21.1 $\pm$ 3.70 (16)	.82 $\pm$ .32 (21)
7	Sacrificed 16 hr after t.r. Saline treatment	13.9 $\pm$ 2.85 (17)	1.23 $\pm$ .36 (19)
C. Tourniquets on both hind-limbs for 4.5 hr anesthetized and teeth clipped.			
8	Sacrificed at 4.5 hr, no t.r.	10.8 $\pm$ 2.59 (18)	2.63 $\pm$ .53 (8)
9	Sacrificed 2 hr after t.r.	16.1 $\pm$ 2.15 (11)	
D. Tourniquets on hind- and fore-limb unilaterally for 5 hr anesthetized and teeth clipped.			
10	Sacrificed 2 hr after t.r.	—	1.68 $\pm$ .58 (11)
11	Sacrificed 16 hr after t.r.	17.9 $\pm$ 3.31 (8)	.73 $\pm$ .19 (13)
12	Sacrificed 16 hr after t.r. Saline treatment	14.7 $\pm$ 3.76 (14)	.96 $\pm$ .27 (14)

\* Tourniquet release.

utilization and excretion of the protein breakdown products.

As can be seen from Table I, occlusion of the limbs *per se* does not cause any change in hematocrit (value obtained at zero time agrees with the normal value). However, release of the tourniquets produces a prompt increase in hematocrit. The elevated blood amino acid and urea levels, observed after tourniquet release, are not due to hemoconcentration alone since the hematocrit increase does not parallel that of the blood amino acid and urea levels.

Exp. 6, 7, 11 and 12 in Table II indicate that the "sparing" effect of saline administration to rats in "shock" is associated with a decrease in the elevation of blood amino acid concentration and in the fall of the adrenal cholesterol content. The nature of the underlying mechanisms of the "sparing" effect of saline administration in reducing mortality is at present obscure. It is quite possible that the administration of saline leads to a correction of the disturbances of body fluids and electrolyte balance brought about by the release of the tourniquets. It is to be noted

that saline therapy does not reverse completely the disturbances in blood amino acid concentration and adrenal cholesterol content by the time the animals were sacrificed.

*Summary and conclusions.* A shock-like state was produced in rats by applying rubber band tourniquets to the limbs for a certain length of time and then releasing them. A progressive increase in blood amino acid and urea nitrogen occurred after the release of the tourniquets. Application of the rubber band *per se* had no effect on these blood constituents but was found to cause a definite stimulation of the adrenal cortex as indicated by a lowering of the adrenal cholesterol content. Release of the tourniquets produced a further decline in the adrenal cholesterol content. The "sparing" effect of 0.9% NaCl administration in lowering mortality of rats during "tourniquet shock" was found to be associated with a decrease in the elevation of blood amino acid concentration and in the fall of the adrenal cholesterol content when compared with untreated animals.

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Conversion of Radioactive Orotic Acid into Pyrimidine Nucleotides of Nucleic Acid by Slices of Rat Liver.\* (18141)

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It has been demonstrated that slices of rat liver have caused the *in vitro* incorporation of radioactive orotic acid into the pyrimidine nucleotides of nucleic acid and not into the purines.

Three milligrams of orotic acid (labeled in the 4 position) were incubated with about 6 g of slices of rat liver for 5 hours at 37°C in a Krebs saline-phosphate buffer of pH 7.4. The solution was saturated with a 95% O<sub>2</sub>: 5% CO<sub>2</sub> mixture at the beginning and nothing was used to diminish bacterial action. The slices were homogenized after preliminary washing and the homogenate precipitated with 10% trichloroacetic acid. The extraction of lipids was then carried out with a 3:1 alcohol and ether solution. The nucleic acid was extracted from the protein with hot 10% NaCl and precipitated with alcohol. All of the dried impure nucleic acid (35 mg containing both r.n.a. and d.n.a.) was hydrolyzed with 0.75 cc of N HCl in a boiling water bath for one hour. This hydrolyzed the purine nucleotides and left the pyrimidine nucleotides unchanged. 0.04-0.06 cc were placed on each of 4 filter paper strips 12 cm wide and run with ascending columns of tertiary butyl alcohol (70% made 0.8 N with HCl, 30% water)(1). After drying, the papers were viewed with an ultraviolet Mineralite. The separated bands on paper were eluted with 0.01 N HCl, and the solutions read with a Beckman ultraviolet spectrophotometer. The maximum absorptions and

TABLE I. Specific Activity of Pyrimidine Nucleotides from Slices of Rat Liver After Incubation with Radioactive Orotic Acid.

Substance	Wt, γ	Counts per min. corrected for background	
		Paper	Per mg base
Uridylic acid	544	37	182
Space	.	1	
Cytidylic acid	674	14	56
Adenine	358	0	0
Guanine	320	0	0
		Resin	
Uridylic acid	1850	153	229
Cytidylic acid	2740	100	103

the ratios of densities (278-262 mμ for cytidylic and uridylic acids; 248-262 for guanine and adenine) were determined in order to identify and quantitate the bases. The separate solutions were evaporated on plates and radioactivity determined with a windowless counter.

Approximately three-fourths of the hydrolysate from the above experiment containing purine bases and pyrimidine nucleotides was adjusted to pH 8 and the guanine precipitate removed by centrifugation. Except for slight modifications, a procedure described by Cohn(2) was then used to separate the remaining components in the supernatant of the hydrolysate on a resin column (Dowex I 400 mesh). The purine bases were removed by thorough washing with water. The cytidylic acid was eluted with 0.002 N HCl, identified and measured with the Beckman spectrophotometer, the solution evaporated and the residue counted. The uridylic acid was eluted with 0.01 N HCl and treated in a similar manner. The data are given in the accompanying table. Another experiment yielded data which confirm this one.

It is apparent that no radioactivity appeared in the purines while highly significant amounts appeared in the pyrimidine nucleo-

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† Atomic Energy Commission Post-Doctoral Research Fellow.

‡ F. B. Workman Fellow from Wellesley College (1949-50).

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tides. Because of the nature of the precursor it is thought that most of the radioactivity was in the pyrimidine ring rather than in the carbons of the sugar.

The radioactivity data obtained from the resin are thought to be better than those from

the paper because there was considerable residue after evaporating solutions from the paper with resulting self absorption. A negligible amount of residue was left after evaporating solutions from the resin.

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## Effects of Vitamin B<sub>12</sub> on Thiouracil Action in Rats.\*† (18142)

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Vit. B<sub>12</sub> has been demonstrated to counteract the deleterious actions of several substances administered in toxic quantities to rats. Thus, it has been shown to overcome the retardation of growth which follows administration of toxic amounts of thyroid powder or thyroprotein(1-4), diethylstilbestrol(4) and lactose(5). In addition, Popper *et al.*(6) have reported that vitamin B<sub>12</sub> can prevent the acute hepatic injury which results from administering carbon tetrachloride to rats.

Thiouracil has been shown to cause a direct depression of thyroid hormone synthesis, thereby stimulating increased production of thyrotrophic hormone by the anterior pitui-

tary with subsequent thyroid hypertrophy (7-8). The inhibition of thyroid function usually results in reductions in growth rate and food intake, although these may also be mediated in part through non-thyroid mechanisms(9). Would vit. B<sub>12</sub> counteract any of these actions of thiouracil?

*Procedure.* Immature female rats of the Carworth strain, divided into 8 uniform groups of 10 each, were fed the following basal ration for 30 days: yellow corn meal, 35%; ground wheat, 25%; linseed oil meal, 10%; whole milk powder, 20%; alfalfa leaf meal, 6%; brewers yeast, 3%; and table salt, 1%. The following substances were added to the basal ration of each group of rats: 1, controls; 2, 40 µg of vit. B<sub>12</sub>§ per kg; 3, 80 µg vit. B<sub>12</sub> per kg; 4, 0.1% thiouracil; 5, 0.1% thiouracil and 40 µg vit. B<sub>12</sub> per kg; 6, 0.1% thiouracil and 80 µg of vit. B<sub>12</sub> per kg; 7, 0.1% thiouracil, and 40 µg of vit. B<sub>12</sub> per kg during last 10 of 30 days; 8, 0.1% thiouracil, and 80 µg of vit. B<sub>12</sub> per kg during last 10 of 30 days. Four hours prior to sacrifice, each rat was injected intraperitoneally with a tracer dose of radio-

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§ Crystalline vitamin B<sub>12</sub> in the form of a triturate of NaCl was kindly furnished by Dr. D. F. Green of the Veterinary Division, Merck and Co., Inc., Rahway, N. J.

TABLE I. Effects of Vitamin B<sub>12</sub> on Thiouracil Action in Rats.

Group	Treatment	Avg body wt		Avg daily food intake per rat, g	Avg thyroid wt		Radioactivity—avg counts per sec.		
		Orig., g	Final, g		Actual, mg	Per 100 g body wt, mg	Per thyroid	Per mg thyroid	Per 100 g body wt
1	Controls	60.3	135.2	9.2	12.5	9.3 ± 1.4*	25.2 ± 3.8*	2.1 ± .6*	18.9 ± 3.5*
2	40 µg B <sub>12</sub>	60.4	147.7	9.7	12.2	8.3 ± 1.7	26.8 ± 6.7	2.3 ± .7	18.2 ± 5.0
3	80 µg B <sub>12</sub>	60.7	153.8	10.1	14.5	9.4 ± 1.3	28.4 ± 7.8	2.0 ± .5	18.8 ± 5.8
4	Thiouracil	61.1	102.7	7.0	51.6	50.3 ± 10.5	3.4 ± 1.1	.07 ± .03	3.4 ± 1.2
5	Thiouracil + 40 µg B <sub>12</sub>	60.1	136.6	8.8	13.6	10.1 ± 3.9	.3 ± .1	.03 ± .02	.2 ± .1
6	Thiouracil + 80 µg B <sub>12</sub>	60.8	144.5	9.4	35.8	24.9 ± 5.4	.5 ± .2	.01 ± .00	.4 ± .2
7	Thiouracil + 40 µg B <sub>12</sub> last 10 days	61.4	105.9	—	51.7	49.3 ± 12.7	3.3 ± 1.3	.08 ± .01	3.2 ± 1.3
8	Thiouracil + 80 µg B <sub>12</sub> last 10 days	60.4	115.8	—	42.8	36.8 ± 5.8	1.4 ± .9	.03 ± .01	1.2 ± .7

$$^* \text{Standard error of mean} = \sqrt{\frac{\sum d^2}{n(n-1)}}$$

active iodine ( $I^{131}$ ) estimated to contain 0.2  $\mu$ c of radioactivity. The thyroids were weighed, dried and counted separately under a thin end window counter. All rats were housed in a constant temperature animal room at 75° F.

*Results.* The data on body growth, food intake and thyroid activity are summarized in Table I. The addition of 40 (Group 2) or 80  $\mu$ g of vitamin B<sub>12</sub> (Group 3) to the ration increased body growth and food intake above that in the control rats (Group 1), but thyroid weight and thyroid uptake of  $I^{131}$  were the same in all 3 groups. Thiouracil (Group 4) drastically reduced body growth, food intake and thyroid concentration of  $I^{131}$ , while thyroid weight was approximately five times that of the controls.

When vit. B<sub>12</sub> was fed in amounts of 40 (Group 5) or 80  $\mu$ g (Group 6) to the thiouracil-treated rats, the inhibitory effects of the drug on body growth were completely overcome throughout the 30-day period. Body growth in group 6 was even greater than in the controls, and food intake was increased in both groups. The ability of thiouracil indirectly to induce thyroid hypertrophy was completely or partially prevented by the vitamin, although thyroid uptake of  $I^{131}$  was even less than in the controls. When 80

(Group 8) but not 40  $\mu$ g of vitamin B<sub>12</sub> (Group 7) was added to the thiouracil ration during the last 10 of the 30-day experimental period, the inhibitory effect of the drug on body growth was partially overcome. Unfortunately, the food-intake data for these 2 groups were not available. While 80  $\mu$ g of vit. B<sub>12</sub> partially counteracted the ability of thiouracil to increase thyroid weight, the concentration of  $I^{131}$  by the thyroids was not increased.

*Discussion.* The data presented here indicate that vit. B<sub>12</sub> can completely overcome the growth-retarding action of thiouracil in female rats. This can be explained partially, if not completely, by the ability of vit. B<sub>12</sub> to increase food consumption in the thiouracil-treated rats. It should be recognized that since the thiouracil was incorporated into the ration, more of the drug was consumed by these vitamin-supplemented rats than by those which received thiouracil only. It is difficult to understand why vit. B<sub>12</sub> should make thiouracil less effective in increasing thyroid weight but more effective in inhibiting uptake of  $I^{131}$  by the thyroid. If the vitamin increased the normal metabolism or excretion of thiouracil in the body, then the lessened ability of thiouracil to induce thyroid hypertrophy could be explained. On the other

hand, since vit. B<sub>12</sub> increased the total thiouracil intake, it would appear logical to expect a greater thyroid inhibition than in the rats which received thiouracil without the vitamin. This would account for the lower concentration of I<sup>131</sup> in the thyroids of the former as compared to the latter rats. On the whole, it seems reasonable to assume that thyroid uptake of a tracer dose of I<sup>131</sup> is a better index of thyroid activity than thyroid weight. The former is believed to reflect direct thyroid secretory ability, whereas the latter reflects an indirect action on the thyroid by pituitary thyrotrophic hormone and does not definitely indicate either increased or decreased thyroid function.

It is interesting to consider whether vit. B<sub>12</sub> can induce normal body growth in hypothyroid rats, as suggested by this study. Thus, vit. B<sub>12</sub> completely counteracted the growth-

retarding action of thiouracil, while the latter was still able to depress thyroid activity. It is possible that vit. B<sub>12</sub> does not require the mediation of the thyroid to exert its anabolic effects on growth.

*Summary.* The effects of crystalline vit. B<sub>12</sub> on thiouracil action was determined in immature female rats for a 30-day period. The vitamin completely counteracted the growth-inhibiting action of thiouracil, and this was accompanied by a considerable increase in food consumption. Although vit. B<sub>12</sub> decreased the thyroid hypertrophy induced by thiouracil, the uptake of radioactive iodine (I<sup>131</sup>) by the thyroids was even less than in the rats which received thiouracil only. It is suggested that vit. B<sub>12</sub> may be able to induce normal growth in hypothyroid rats.

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## Effects of Vitamin B<sub>12</sub> on Normal Thyroid Function in Rats.\*† (18143)

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Vitamin B<sub>12</sub> can counteract the retardation of growth in young rats which results from administering toxic amounts of thyroid powder or thyroprotein(1-4). The mechanism by which vitamin B<sub>12</sub> achieves this effect has not been explained, although Monroe and

Turner(5) presented data indicating that the vitamin increases the catabolism of administered thyroxine in chicks. If this is so, then vitamin B<sub>12</sub> may also favor the catabolism of endogenous thyroid hormone, in which case pituitary thyrotrophic hormone secretion would be stimulated with a subsequent increase in thyroid secretion rate. It was considered of interest therefore, to determine whether or not the administration of vitamin B<sub>12</sub> would alter normal thyroid function in immature rats.

*Procedure.* Young male and female rats of the Carworth strain were placed on experiment for 20 and 30 days respectively. With the exception of 2 groups, all rats were fed the following basal ration: yellow corn meal, 35%; ground wheat, 25%; linseed oil meal, 10%; whole milk powder, 20%; alfalfa leaf meal, 6%; brewers yeast, 3%; and table salt,

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VITAMIN B<sub>12</sub> ON NORMAL THYROID FUNCTIONTABLE I. Effects of Vitamin B<sub>12</sub> on Normal Thyroid Function in Rats.

Group	Treatment	Avg body wt		Avg thyroid wt		Radioactivity—avg counts per sec.		
		Orig., g	Final, g	Actual, mg	Per 100 g body wt, mg	Per thyroid	Per mg thyroid	Per 100 g body wt
Female rats—30 days trial								
1	Controls	60.3	135.2	12.5	9.3 ± 1.4*	25.2 ± 3.8*	2.1 ± .6*	18.9 ± 3.5*
2	40 µg B <sub>12</sub>	60.4	147.7	12.2	8.3 ± 1.7	26.8 ± 6.7	2.3 ± .7	18.2 ± 5.0
3	80 µg B <sub>12</sub>	60.7	153.8	14.5	9.4 ± 1.3	28.4 ± 7.8	2.0 ± .5	18.7 ± 5.8
Male rats—20 days trial								
4	Controls	57.2	117.2	10.9	9.4 ± 1.3	9.0 ± 1.5	0.7 ± .1	6.9 ± 1.2
5	80 µg B <sub>12</sub>	56.0	139.8	11.8	8.3 ± 1.8	10.0 ± 4.5	1.0 ± .4	7.1 ± 2.2
6	Controls†	56.0	136.1	12.7	9.4 ± 2.1	16.6 ± 9.6	1.3 ± .5	12.4 ± 7.4
7	80 µg B <sub>12</sub> †	56.1	142.1	12.6	8.7 ± 1.9	19.1 ± 6.0	1.6 ± .5	13.7 ± 4.5
Male rats—20 days trial								
8	Controls	55.6	122.5	9.2	8.5 ± 0.6	26.0 ± 2.5	2.8 ± .2	21.6 ± 2.2
9	40 µg B <sub>12</sub>	56.7	128.8	11.2	8.8 ± 0.7	21.9 ± 2.0	2.0 ± .1	17.0 ± 1.4
10	80 µg B <sub>12</sub>	56.3	140.2	11.0	8.0 ± 0.5	23.4 ± 2.1	2.1 ± .2	16.7 ± 1.4
11	.16% thyro-protein	56.5	97.7	6.4	6.5 ± 0.7	0.06 ± 0.05	0.01	0.06 ± 0.04
12	.16% thyro-protein + 40 µg B <sub>12</sub>	56.2	126.0	6.2	4.8 ± 0.4	0.13 ± 0.08	0.02	0.10 ± 0.06

$$* \text{ Standard error of mean} = \sqrt{\frac{\sum d^2}{n(n-1)}}$$

† Received modified diet in which casein, corn oil and cerelose were substituted for whole milk powder.

1%. Vit. B<sub>12</sub>§ was mixed into the ration in amounts of either 40 or 80 µg per kg of feed. For 2 groups of male rats, 0.16% thyroprotein¶ was incorporated in the ration. Four hours prior to sacrifice, each rat was injected intraperitoneally with a tracer dose of I<sup>131</sup> estimated to contain 0.2 µc of radioactivity. The thyroids were carefully removed, weighed and placed in small copper discs. After allowing the thyroids to dry overnight, the radioactivity of each was counted separately under a thin mica-end window counter. All rats were housed in a constant temperature animal room at 75°F.

**Results.** The data are summarized in Table I. The first experiment was performed on 3 groups of female rats. It can be seen that the addition of either 40 (group 2) or 80 µg of vit. B<sub>12</sub> (group 3) to the ration increased the final body weights of the rats above that

§ Crystalline vit. B<sub>12</sub> in the form of a triturate of NaCl was made available through the courtesy of Dr. D. F. Green of the Veterinary Division, Merck and Co.

¶ Thyroprotein (Protamone) was made available through the courtesy of Cerophyl Laboratories, Kansas City, Mo.

of the controls (group 1). Apparently the basal ration did not contain an amount of B<sub>12</sub> sufficient for optimal growth. Vit. B<sub>12</sub> did not alter the thyroid weight of these rats when calculated on a body weight basis. The uptake of I<sup>131</sup> by the thyroids of all three groups was the same whether calculated on a thyroid, per mg of thyroid or on a body weight basis.

The results of the next experiment on 4 groups of male rats are essentially in agreement with the data on female rats. The inclusion of 80 µg of vit. B<sub>12</sub> per kg of ration did not alter thyroid weight or uptake of I<sup>131</sup>. It is notable that the controls (group 6) which received the modified diet gained more body weight than the controls (group 4) fed the basal ration.

Vit. B<sub>12</sub> did not alter thyroid function in the next experiment, as can be seen by comparing the data in groups 9 and 10 with group 8. The small differences in concentration of I<sup>131</sup> between the thyroids of the three groups are not statistically significant. The administration of thyroprotein (group 11) significantly reduced the final average body and thyroid weights when compared with the

controls (group 8). The thyroids of these rats were apparently nonfunctional, as indicated by the virtual absence of  $I^{131}$  in the glands. In fact, the radioactivity in these thyroids did not differ statistically from that present in the atmosphere (background). Although the administration of 40  $\mu g$  of vit.  $B_{12}$  (group 12) completely overcame the inhibitory effects of the thyroprotein on growth, it did not counteract the effects of the latter on thyroid activity.

**Discussion.** These experiments indicate that the administration of vit.  $B_{12}$  does not alter normal thyroid function in young rats, even when given in amounts sufficient to increase body growth. Apparently, vit.  $B_{12}$  does not affect the metabolism or excretion of endogenous thyroid hormone, since such changes would have altered thyroid activity and uptake of  $I^{131}$ . It seems reasonable to conclude that vit.  $B_{12}$  can increase body growth in rats independently of the thyroid.

On the whole, these data also do not sup-

port the view that vit.  $B_{12}$  increases the turnover within the body of administered thyroid materials, since the vitamin did not at all overcome the thyroid-inhibiting action of thyroprotein. However, it is possible that the dose of thyroprotein required to completely inhibit thyroid activity was lower than the amount given, and hence its increased metabolism by vit.  $B_{12}$  was not reflected by the thyroid.

**Summary.** Crystalline vit.  $B_{12}$  was fed to immature rats of both sexes in order to determine whether the vitamin could alter thyroid function in normal or thyroprotein-treated rats. The growth rate of the rats supplemented with the vitamin was increased above that of the normal or thyroprotein-treated controls, but there was no significant effect on thyroid weight or uptake of  $I^{131}$ . It is concluded that vit.  $B_{12}$  does not alter normal thyroid activity in rats.

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### Studies on the Destruction of Red Blood Cells. VIII. Molecular Orientation in Sickled Cell Hemoglobin Solutions.\* (18144)

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Sherman(1) reported "without interpretation" the observation that "under the polarizing microscope characteristic sickle cells exhibit a definite birefringence". Since that time, several writers have used this finding as evidence that the hemoglobin molecules assume an orderly arrangement in sickled erythrocytes(2,3). When hemoglobin was

removed from the red cells, Ponder(2) demonstrated that the "ghosts" would not become sickle shaped on exposure to low oxygen tensions. Recent studies by Pauling *et al.* (3,4) have shown that certain physical and chemical properties which differentiate sickle cell hemoglobin from normal hemoglobin result from an alteration in the structure of the globin portion of the molecule. Human deoxygenated hemoglobin is said to be less soluble than the oxygenated form, suggesting that the phenomenon of sickling might be related to incipient crystallization of the

\* Supported by the Medical Division of the Atomic Energy Commission, Contract No. AT (30-1)-675.

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2. Ponder, E., *Hemolysis and Related Phenomena*, New York, Grune and Stratton, 1948, p. 145.

3. Pauling, L., Itano, H. A., Singer, S. J., and Wells, I. C., *Science*, 1949, v110, 543.

4. Pauling, L., Itano, H. A., Wells, I. C., Schroeder, W. A., Kay, L. M., Singer, S. J., and Corey, R. B., *Science*, 1950, v111, 459.

hemoglobin(5). Rebuck *et al.*(6) have observed that in the early stages of sickling the intracellular hemoglobin forms anisotropic aggregates with intense peripheral spiculation "suggestive of incipient crystallization". Ponder(2) has proposed that, in the state of oxygen unsaturation, the intracellular hemoglobin molecules assume an orderly or "paracrystalline" arrangement which results in the various shape alterations by means of "expansive" or "turgor-producing" forces arising from a physico-chemical interaction of the paracrystalline hemoglobin with the enveloping ultrastructure. However, aside from the fact that sickling is basically dependent upon the removal of oxygen from the hemoglobin, these few observations constitute the only *direct* evidence concerning the physical basis of the sickling process.

In the present study, Sherman's observation (1) of the birefringence of sickled cells was readily confirmed by examining intact cells under the polarizing microscope after sickling had been induced by any of the methods usually employed(7). Normal intact erythrocytes did not become birefringent when treated in the same manner. It was therefore considered desirable to establish, if possible, further evidence for molecular orientation or alignment of the abnormal hemoglobin as a basis for the sickling phenomenon. Accordingly, certain observations were made employing stroma-free solutions of hemoglobin in distilled water, prepared by a modification of the method described by Drabkin(8).

*Viscosity measurements* were made at 37.5°C with the Ostwald capillary viscometer, using concentrations of hemoglobin in distilled water ranging from 15 to 25 g/100 ml. When the oxygen saturation of sickle-cell hemoglobin was decreased progressively from 100 to the vicinity of 10%, marked increases in viscosity occurred in the lower range of oxygen saturations. Indeed, the more concentrated hemoglobin solutions assumed a

semi-solid gel-like state. All such increases in viscosity could be readily reversed by reoxygenation of the hemoglobin solutions, and the cycle could be repeated as desired. However, no increase in viscosity occurred when the concentration of sickle cell hemoglobin was decreased to 10 g/100 ml or less. Solutions of normal hemoglobin, in concentrations ranging from 15 to 25 g/100 ml, showed no changes in viscosity when treated by the same methods. The increased viscosity of solutions of deoxygenated hemoglobin from sickle cell anemia is evidence for a grouping of the individual hemoglobin molecules such that asymmetric aggregates are formed, resulting in increased internal friction(9) of the solution. The absence of change in viscosity of solutions of less than 10 g/100 ml suggests that molecular interactions or attracting forces are effective only at limited distances. On the average, the distance between two hemoglobin molecules at closest approach in the red cell, as determined by x-ray diffraction, is of the order of magnitude of two water molecules(5). Furthermore, the increases in viscosity take place only at low saturations of oxygen, indicating that molecular interactions are possible only when oxygen is absent from the hemoglobin unit, thus in some way activating or uncovering intermolecular attracting forces.

*Microscopic observations* of wet preparations at a magnification of 1000 $\times$  showed no differences between oxygenated or deoxygenated solutions of normal hemoglobin and of oxygenated solutions of sickle cell anemia hemoglobin ranging from 15 to 25 g/100 ml. However, when the solutions of sickle cell hemoglobin were in the deoxygenated and viscous state, spindle-shaped bodies varying in length from 1 to 15  $\mu$  were observed in the wet preparations examined under a sealed coverslip (Fig. 1). These bodies proved to be birefringent when examined under the polarizing microscope and thereby showed the requisite characteristics of tactoids(10). They disappeared upon reoxygenation of the hemo-

5. Granick, S., *Blood*, 1949, v4, 404.
6. Rebuck, J. W., Sturrock, R. M., and Monaghan, E. A., *Fed. Proc.*, 1950, v9, 340.
7. Daland, G. A., and Castle, W. B., *J. Lab. & Clin. Med.*, 1948, v33, 1082.
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TABLE I. Comparison of Normal and Sickle-Cell Hemoglobin in Stroma-Free Solutions and in Intact Erythrocytes.

Sample	Hemoglobin solutions — stroma-free				Intact erythrocytes	
	Viscosity relative to distilled water		Birefringent tactoids Oxygen saturation (%)	100	Birefringence Oxygen saturation (%)	
	Oxygen saturation (%)	100		10	100	10
Normal hemoglobin						
15.1 g/100 ml	4.34	3.67	Absent	Absent	Absent	Absent
25.0 " "	46.1	47.2	"	"	"	
Sickle cell anemia hemoglobin						
6.9 g/100 ml	2.06	1.88	"	"	"	
15.2 " "	7.60	37.1	"	"	Present	"
23.5 " "	41.1	Semi-solid gel-like state	"	"		Present

globin solution and were reformed when the oxygen was again removed.

The tactoid form is characteristic of an orderly grouping of long, thin, rod-like particles which are arranged parallel and equidistant to each other(11). Similar bodies have been described in solutions of the asymmetric tobacco mosaic virus and in some colloidal suspensions(11). Since the individual hemoglobin molecule is not markedly asymmetric ( $57\text{\AA} \times 57\text{\AA} \times 34\text{\AA}$ )(5), the formation of tactoids by the oxygen unsaturated hemoglobin of sickle cell anemia is evidence of a specific arrangement or linkage of the individual molecules with the formation of long chains of hemoglobin elements and the subsequent alignment of these elements into an anisotropic grouping. Evidence that this process occurs in the intact sickle cell is found in the observation that the sickled forms of intact cells are birefringent(1,6). The intact cells contain approximately 30 g/100 ml of hemoglobin(15), a concentration ample for tactoid formation of the hemoglobin when in solution(5). It is probable, therefore, that the sickled erythrocyte is in essence a hemoglobin tactoid thinly veiled and somewhat distorted by the cell membrane (Fig. 2).

In conclusion, it would appear that beginning with the genetically(12) abnormal hemoglobin molecule(3) and extending to the

clinical manifestations, the following sequence of events would be adequate to explain the major aspects of the pathologic physiology of sickle cell disease. The alignment and parallel aggregation of the molecules of deoxygenated hemoglobin derived from the red cells of patients with sickle cell disease are manifested as increased viscosity of hemoglobin solutions and the formation of hemoglobin tactoids. These effects take place in solutions at concentrations comparable to those of the intracellular hemoglobin. The resemblance of tactoids to sickled erythrocytes is so striking that the sickled red cell in all probability is essentially a membrane-covered hemoglobin tactoid. Due entirely to the sickled form of the erythrocytes, the viscosity(13) of the whole blood and the mechanical fragility(14) of sickled cells are significantly increased at low oxygen tensions. The increase in viscosity appears to explain the multiple venous thromboses, and the increase in mechanical fragility may largely explain the hemolytic anemia—phenomena which are characteristic of the active disease. Thus, in sickle cell anemia (genetically homozygous)(12), the erythrocytes contain 100% abnormal hemoglobin(3). Sickling and increases in viscosity and in mechanical fragility of the red cells occur within the range of oxygen saturations of venous blood

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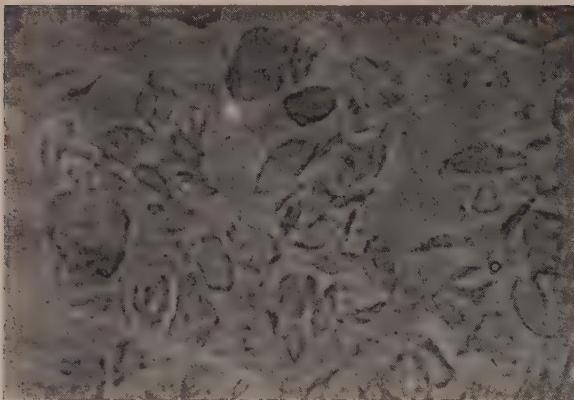


FIG. 1.

Hemoglobin tactoids formed in stroma-free solutions of deoxygenated sickle cell anemia hemoglobin. (Phase microphotography  $\times 375$ .)



FIG. 2.

Sickled erythrocytes in oxygen unsaturated whole blood from a patient with sickle cell anemia demonstrating the similarities in shape to that of tactoids formed in stroma-free solutions of their deoxygenated hemoglobin. (Phase microphotography  $\times 375$ .)

(15). When transfused into normal recipients, sickle cell anemia erythrocytes do not long survive(16). In sickle cell trait (genetically heterozygous)(12), only 25 to 44% of the hemoglobin is abnormal(4). The condition is without clinical manifestations and sickling and increases in viscosity and in mechanical fragility of the red cells can be

produced only by oxygen saturations well below those usual for venous blood(15). When transfused into normal recipients, sickle cell trait erythrocytes survive normally(16). Thus, the clinical and pathologic manifestations of sickle cell disease apparently derive from a single basic abnormality: the orderly molecular orientation of the peculiar hemoglobin that occurs in concentrated solutions at the reduced oxygen saturation of normal venous blood.

*Summary.* 1. In the oxygen unsaturated

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16. Callender, S. T. A., Nickel, J. F., and Moore, C. V., *J. Lab. and Clin. Med.*, 1949, v34, 90

state the abnormal sickle cell hemoglobin molecules undergo orderly orientation, forming—by specific linkage of the individual molecules—long chains of hemoglobin elements. Subsequent parallel alignment of these elements results in birefringent tactoids.

2. The birefringent sickled erythrocyte is in all probability a membrane-covered hemoglobin tactoid.

3. The clinical and pathologic manifestations of sickle cell disease apparently follow as a consequence of the effects of the abnor-

mal hemoglobin molecules upon the physical behavior of the erythrocytes.

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## Effect of Cortisone and ACTH on Eosinophils and Anaphylactic Shock in Guinea Pigs. (18145)

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Adrenalectomy has been reported to increase the susceptibility of guinea pigs(1) and rats(2,3) to anaphylactic shock. On the other hand, administration of adrenocortical extracts has been found to confer some protection to guinea pigs and dogs against anaphylactic shock(4,5) and some protection to rats against peptone shock(6). Leger, Leith and Rose(7) have found, however, that the administration of 3.5 to 4 mg of adrenocorticotropic hormone (ACTH) to sensitized guinea pigs 6 to 8 hours prior to giving the challenging dose of the antigen did not influence the course of the ensuing anaphylactic reaction. Since the effect of cortisone on anaphylactic shock in guinea pigs had not been studied and since some protective action

of ACTH might conceivably have been missed by Leger, Leith and Rose owing to the dosage and time schedule they followed, this investigation was undertaken to extend their study and at the same time to determine whether administration of cortisone would affect anaphylactic shock in guinea pigs and the number of eosinophils in their blood.

**Methods.** Thirty-seven male and 39 female guinea pigs each weighing between 250 and 300 g were sensitized by 4 consecutive daily subcutaneous injections of 0.5 ml of egg-white solution. The egg-white solution was prepared by diluting the white of fresh eggs with physiologic saline solution in the proportion of 1:5 and thoroughly shaking the mixture until the gelatinous character of the egg white had disappeared. The mixture was then strained through gauze and a clear solution, subsequently referred to as egg-white solution, was obtained. Eight to 12 weeks after the last sensitizing injection a challenging injection that varied from 1.0 ml to less than 0.1 ml of egg-white solution was administered intravenously. The body weights of the animals had by then approximately doubled. Eosinophil counts were performed

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6. Ingle, D. J., *Am. J. Physiol.*, 1944, v142, 191.
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on drops of blood obtained by needle puncture of the marginal vessels of the ear. The blood was drawn directly into a leukocyte pipet, the diluting fluid and the counting chamber used being those adopted by Thorn, Forsham, Prunty and Hills(8) in their modification of Dunger's method(9). A saline suspension of cortisone acetate containing 25 mg per ml, kindly supplied by Merck Co., Inc., Rahway, N. J., was used throughout this study. The lyophilized adrenocorticotrophic hormone (ACTH) employed in the study, kindly provided by the Armour Laboratories, Chicago, Ill., had a biological potency of 100% of Armour La-I-A standard. The amounts needed each day were weighed out, then dissolved in physiologic saline solution and injected within 30 minutes. Cortisone and ACTH in doses of 30 and 15 mg respectively were injected in volumes of 1.2 ml and this same volume of physiologic saline solution was used in the control series of animals. All of the injections were given intramuscularly.

Tests of the effects of the hormone on anaphylactic shock were carried out on groups of 6 to 15 sensitized animals. In each group approximately equal numbers of animals were given cortisone, ACTH or physiologic salt solution. Those receiving the salt solution alone served as controls. Nine to 204 hours after receiving the cortisone or saline and 9 to 88 hours after receiving ACTH, the animals were given a challenging injection of egg-white solution. Twenty-seven of the guinea pigs received cortisone, 26 ACTH and 23 were given salt solution only. The distribution of males and females was approximately equal in each group. Eosinophil counts were carried out on 21 of the animals in each of the test groups and on 18 of the animals in the control series. Control eosinophil counts were made during the 3 or 4 days just prior to the hormone or saline injections. The majority of the animals had two or more such counts. From one to 9 counts on each

animal were then done at various intervals following the injections. For each guinea pig, the number of eosinophils found after the hormone or saline injections was expressed as a per cent of the mean of its control or preinjection counts. An attempt was made to test the susceptibility of the animals to anaphylactic shock during the early, mid and late periods of the eosinopenia which followed the injections of hormone. After death from anaphylactic shock the lungs of all animals were carefully examined.

*Results. Effect of cortisone and ACTH on the number of eosinophils in the blood. Control eosinophil counts.* The number of eosinophils in the blood of 28 males and 32 females was counted before hormone or saline injections (Table I). The mean of the 56 preinjection counts made on the 28 males was 45 eosinophils per cmm with a standard error of the mean of  $\pm 6$  cells per cmm (the mean of the first count made on the males was  $45 \pm 11$ ). The mean number of eosinophils observed in the 64 preinjection counts made on the 32 females was 155 per cmm with a standard error of the mean of  $\pm 11$  (the mean of the first counts made in the females was  $148 \pm 17$ ). Because all of these animals had been sensitized and many of the females

TABLE I. Eosinophil Counts in Guinea Pigs.

Group	Mean body wt, g	Counts		No. of	
		Animals	Counts	Animals	Mean No. eosino-
Males					phil per mm <sup>3</sup>
Sensitized	566	28	56	45 $\pm$	6
Nonsensitized	393-844†	3	29	39 $\pm$	6
Females					
<i>Sensitized</i>					
Primigravida	544	32	64	155 $\pm$	11
<i>Nonsensitized</i>					
Virgin	392-758†	10	82	105 $\pm$	7
Nongravid multipara	1,098	10	25	165 $\pm$	19
Gravid multipara	1,368	7	16	84 $\pm$	12

\* Values following the  $\pm$  signs are standard errors of the means.

† Counts were made repeatedly on these animals over a period of 4 months during which the body weights approximately doubled.

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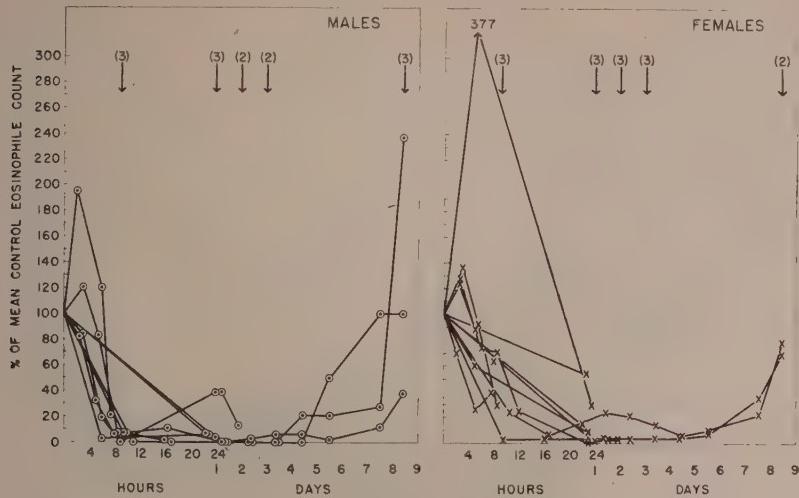


FIG. 1.

Effect of injection of 30 mg of cortisone on the number of eosinophils in the blood of guinea pigs. The arrows indicate the times at which the susceptibility of the animals to anaphylaxis was tested and the numerals in parentheses are the number of animals tested on each occasion.

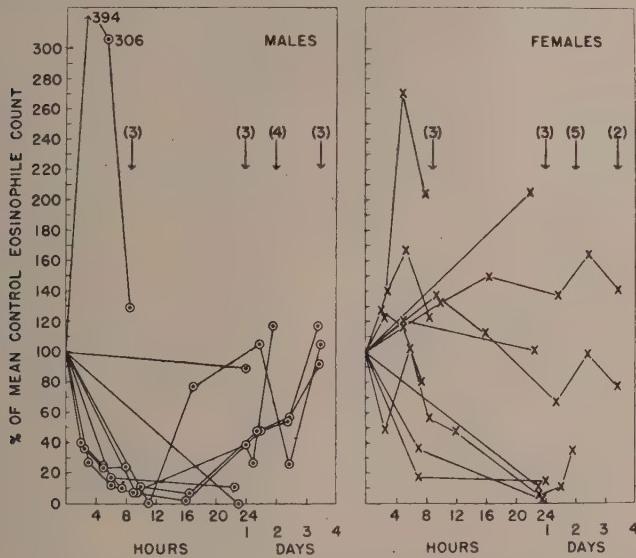


FIG. 2.

The effect of injection of 15 mg of ACTH on the number of eosinophils in the blood of guinea pigs. The arrows indicate the times at which the susceptibility of the animals to anaphylaxis was tested and the numerals in parentheses are the number of animals tested on each occasion.

were found to be pregnant, a series of eosinophil counts were done on 10 nulliparous non-sensitized females and a series of 25 counts

were made on 3 unsensitized males. The mean number of eosinophils per cmm of blood was  $105 \pm 7$  in the females and  $39 \pm 6$  in

## CORTISONE, ACTH ON EOSINOPHILS, ANAPHYLACTIC SHOCK

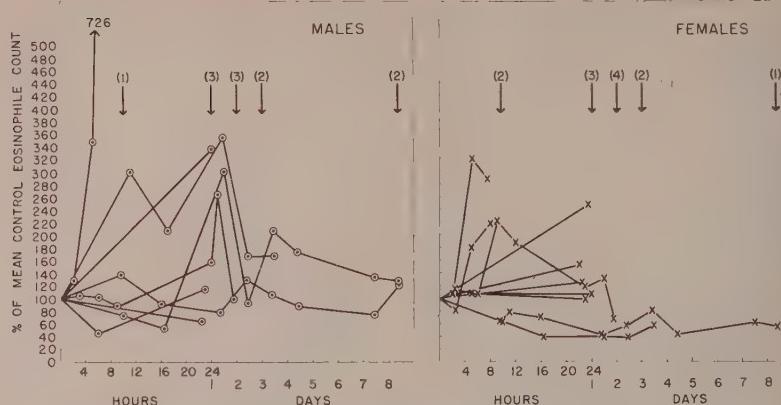


FIG. 3.

The effect of injection of saline solution on the number of eosinophils in the blood of guinea pigs. The arrows indicate the times at which the susceptibility of the animals to anaphylaxis was tested and the numerals in parentheses are the number of animals tested on each occasion.

the males. The mean number of eosinophils in the males was therefore significantly different from that in the females.

*Effect of cortisone.* The injection of 30 mg of cortisone produced a pronounced reduction in the number of eosinophils in the blood of all the animals tested. Counts made 8 to 12 hours after injection of the cortisone into the male animals were all less than 10% of the preinjection values (Fig. 1). In the females the decline was more gradual, ranging at the 8 to 12 hour period between about 70% and 2% of the control counts although by the end of the first 24-hour period most of the counts in the female animals were below 20% (Fig. 1). In 4 of the 5 animals in which counts were made for 8 days or more, the eosinopenia persisted for 7 days, after which the counts approached normal.

*Effect of ACTH.* Eosinophil counts were made on 10 male and 11 female animals after the injection of ACTH. In 7 of the 10 males the eosinophils were reduced in number within 12 hours to less than 20% of preinjection values. The eosinopenia may have been missed in 2 of the 3 animals because a count was not made until 23 to 24 hours after the injection (Fig. 2). The duration of the eosinopenia was much shorter after the ACTH than after the cortisone injections, the counts having returned to normal 24 to 72 hours after

the injections of ACTH (Fig. 2). ACTH did not have a consistent effect on the number of eosinophils in the blood of the female animals. In 4 animals there was a definite eosinopenia while in the remaining 7 there was an increase or no consistent change in the number of eosinophils (Fig. 2).

*Injections of saline solution.* The injections of saline solution did not produce a consistent change in the number of eosinophils in the blood of the male or female animals. Approximately as many animals showed a rise in numbers of eosinophils as displayed a fall and none had a reduction in number below 39% of the control values (Fig. 3).

The abdomens of about half the females were examined at necropsy and in every instance a gravid uterus was found. Since the male and female animals were mixed in the cages it is likely that nearly all, if not all, of the females studied were pregnant when the tests were made. Because the females in the foregoing series did not exhibit a consistent eosinopenia after ACTH and because most of them were pregnant the effect of ACTH was tested on an additional series of 7 pregnant and 10 nonpregnant unsensitized females (Table I). All of these had been pregnant previously and were larger than those of the other groups. Their body weights ranged from 0.9 to 1.4 kg and the amount

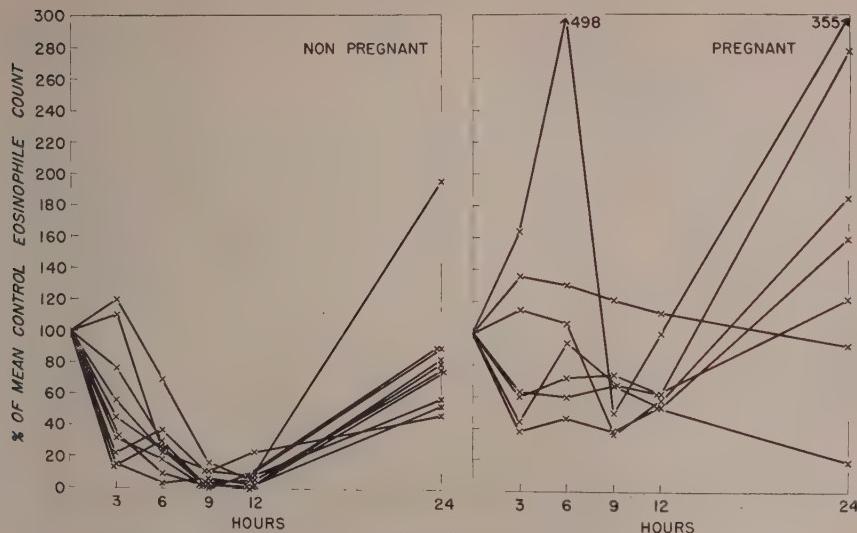


FIG. 4.

The effect of injection of ACTH on the number of eosinophils in the blood of pregnant and nonpregnant female guinea pigs.

of ACTH given was therefore increased proportionally. It was injected after 2 or 3 daily control eosinophil counts had been made. The counts were then repeated 3, 6, 9, 12, and 24 hours after the injection. The mean of 25 control counts on the nonpregnant animals was  $165 \pm 19$  and that of 16 counts on the 7 pregnant animals was  $84 \pm 12$ . After the injection of ACTH the nonpregnant animals showed a consistent eosinopenia similar to that obtained in the males. The pregnant females did not show a consistent change in numbers of circulating eosinophils (Fig. 4). It was concluded that pregnancy abolishes the eosinopenic response to ACTH in female guinea pigs.

*Effect of cortisone and ACTH on anaphylactic shock.* The challenging injections of egg-white solutions were given to the animals 9, 24, 48, 73, 88 and 204 hours after the hormone or saline injections (see arrows in Fig. 1, 2 and 3). This timing was carried out in an attempt to test the sensitivity of the animals to the egg-white solution while the number of eosinophils in the blood was declining, when the eosinopenia was maximal and during the return of the counts to the control values. All the animals experienced severe grades of anaphylactic shock a few

moments after receiving the egg white. Seventy-four of the 76 injected died within 5 minutes and the remaining 2 succumbed within 20 minutes. The animals dying promptly all showed the voluminous lungs typical of anaphylaxis. The 2 in which death was delayed showed patchy emphysema. Therefore the administration of cortisone and ACTH in these experiments did not protect the animals against anaphylactic shock.

*Comment.* The eosinopenia produced by ACTH in the male and nonpregnant female guinea pigs is similar to that observed by Hills, Forsham and Finch(10) in human beings. The eosinopenia produced by cortisone in the guinea pigs was, on the other hand, more pronounced and consistent than the changes produced by this hormone in the number of circulating eosinophils in human beings (11,12). The duration of action of the single

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30 mg injection of cortisone was approximately one week. This prolonged effect was probably due, in part at least, to the insolubility of the compound and its resultant slow absorption, because upon examination the material could often be seen at the site of injection 2 or 3 days and sometimes longer after it had been given.

The failure of ACTH to affect anaphylactic shock in guinea pigs confirms the earlier observations of Leger, Leith and Rose. Employing a larger dose of ACTH in the present study and testing at various intervals after the administration of the hormone did not affect the results. Wolfram and Zwemer(4) noted a brief period from  $2\frac{1}{2}$  to  $5\frac{1}{2}$  hours after the administration of adrenocortical extract during which sensitized guinea pigs were

apparently partially protected against injections of the sensitizing agent. In the present study tests for sensitivity were not made during this early period.

**Summary.** Guinea pigs were used throughout this study. The number of eosinophils in the blood of males was significantly lower than that in females. Cortisone reduced the number of eosinophils in the blood of males and females. ACTH produced a pronounced eosinopenia in males and nonpregnant females. Pregnancy abolished the eosinopenic effect of ACTH. Neither cortisone nor ACTH had any effect upon the degree of anaphylactic shock produced in guinea pigs by the intravenous injection of the agent to which the animals had been sensitized.

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### Effect of Specific Antibody on the Metabolism of *Serratia marcescens*.\* (18146)

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From time to time studies have been carried out to determine any possible effect which specific antisera might have on the growth and metabolism of various bacteria. We shall not attempt at this time to survey the literature on this subject which has been adequately covered by Oldfelt(1) and Sevag (2). Suffice it to say, with few exceptions, there is agreement that when a bacterium comes in contact with its specific antiserum little or no change can be demonstrated in its metabolism as measured by oxygen consumption, providing lysis of the organism does not occur. Because of some preliminary ex-

periments we have felt that this problem should be re-studied, using simple substrates, purified (ethanol precipitated) antibody and suspensions of resting (washed) organisms.

**Experimental.** Rabbits were injected intravenously 6 times at 2- or 3-day intervals with emulsions of live *S. marcescens* prepared from cultures grown for 24 hours on pancreatic digest agar. Twelve days after the last injection the animals were bled from the heart. Each serum was inactivated at 60°C for 3 minutes and was then titrated against live organisms. After finding the sera to be active (titer: 1-20,480 to 1-40,960) all were pooled. A portion of this was frozen and used as necessary. The remainder was treated with ethanol to obtain the gamma globulin fraction as described by Cohn *et al.*(3). Normal rabbits were bled; after inactivation

\* This investigation was aided in part by grants from the John and Mary Markle Foundation and the Division of Research Grants and Fellowships of the National Institute of Health, United States Public Health Service.

1. Oldfelt, C-O., *Acta Med. Scand.*, 1942, Supp. 132.

2. Sevag, M. G., Immuno-Catalysis, Chas. C. Thomas, Publisher, Springfield, 1945.

3. Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, 1946, v68, 459.

some of the serum was frozen; the rest was treated with ethanol.

To obtain suspensions of resting organisms, 9-hour cultures grown on trypticase-soy<sup>†</sup> agar were washed from the medium with phosphate buffer (pH 7.4). They were centrifuged, the supernatants decanted, new buffer added and the tubes recentrifuged. This process was repeated 3 times. Three cc of buffered Krebs-Ringer solution containing the substrate were placed in the main chamber of each Barcroft-Warburg reaction vessel together with .1 cc of an appropriate suspension of organisms. The concentration of bacilli giving optimum O<sub>2</sub> consumption had to be determined by trial and error; after this was obtained it was standardized against a suspension of BaSO<sub>4</sub> so that density of organisms could be repeated at will. One-tenth cc of antiserum and normal serum or various concentrations of precipitated antibody and normal gamma globulin were placed in the arms of respective flasks. Two-tenths cc 10% KOH were placed with filter paper in the center cups. Observations were made for periods up to 6 hours. A variety of substrates were studied: glucose, succinate, malate, mannite, sorbitol, inulin. With all of these substrates no differences in O<sub>2</sub> uptake could be found between those flasks containing antibody and those containing normal serum.

Before abandoning the experiment it was decided to study the effect, if any, of antibody on the growing organisms. Accordingly, an 8-hour culture grown in pancreatic digest broth was diluted 1-500 with trypticase soy broth; 3 cc of this were placed in the main chamber of reaction vessels. Antibody or normal serum were added to respective side arms, while 10% KOH was placed in the center wells. With this dilution of organisms there was very little uptake of O<sub>2</sub> during the first 2 hours. By the third hour it was apparent that the flasks containing antibody were showing an increased O<sub>2</sub> uptake and the difference became even more marked during the ensuing 2 hours. In the following hours as the concentration of organisms in each

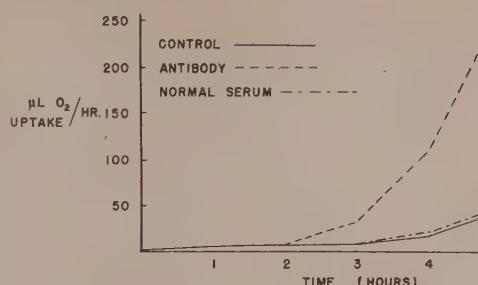


FIG. 1.

Oxygen uptake in micro liters per hour at varying times. Control flask contained medium and bacteria; normal serum flask contained medium, bacteria and .1 cc normal rabbit serum; antibody flask contained medium, bacteria and .1 cc rabbit antiserum for *S. marcescens*.

group of flasks increased the oxygen uptake became too large to measure. A typical experiment is shown graphically in Fig. 1. These results have been uniformly consistent.

The difference in rate of O<sub>2</sub> uptake may be increased if 100% O<sub>2</sub> rather than 20% O<sub>2</sub> makes up the gas phase in the flask. If CO<sub>2</sub> is not absorbed the difference is negligible. There is a more rapid fall in pH in the medium containing antibody; this difference, though not large (0.2 pH in 4 hours) has been consistent. In addition, an increased glycolysis as evidenced by a fall in concentration of reducing substances in the media containing antibody has been noted. We have not been able to detect any difference in O<sub>2</sub> uptake when washed organisms have been utilized with trypticase-soy broth as substrate. This, we feel would seem to indicate that growing organisms are necessary to elicit the phenomenon. Furthermore, no differences have been elicited when growth has been inhibited by appropriate concentrations of streptomycin.

Because of the possibility that lysis might be taking place and might account for the phenomenon we have deliberately added guinea pig complement to the system, since the antiserum had been inactivated. This had no effect. Washed organisms were suspended in water at 37°C in an attempt to produce autolysis; after 24 hours the supernatant fluid was tested against the medium. There was no O<sub>2</sub> uptake whatsoever. In addition, a heavy suspension of organisms was

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mechanically ground with carborundum (grit 600) in a glass tube and pestle. The supernatant showed no differences with and without added antibody. Furthermore, a paste of organisms was frozen at -78°C and placed in a cylinder at the same temperature; a piston was struck with a heavy weight in order to disintegrate the bacteria. Smears showed very few intact organisms remaining after such treatment. The supernatant of this material as well as the disintegrated residue itself was inactive.

Although certain hypotheses come to mind, at the present time we do not feel a discussion of this phenomenon is warranted. It should be pointed out that experiments heretofore

reported have ordinarily employed 24-hours cultures; no investigations have been previously carried out using organisms during the early stages of their growth. Experiments are now in progress with other organisms. In addition synthetic media of known composition are being investigated in an attempt to elucidate this phenomenon more fully.

**Summary.** When growing bacteria, *S. marcescens*, are placed in contact with their specific antibody and with normal serum or gamma globulin there is an increased O<sub>2</sub> consumption by the former over the latter, particularly when CO<sub>2</sub> is absorbed.

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### Studies on the Nutrition of *Leptospira canicola*.\* (18147)

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The metabolism of various amino acids, carbohydrates, vitamins and salts utilized by pathogenic leptospira has been investigated by a number of workers(1-8) but there has been no report of a chemically-defined medium adequate to support growth of this group of

\* This work was aided by grants from the University of California, the National Institutes of Health (U. S. Public Health Service), and a grant to one of us (M.S.D.) from the American Cancer Society, through the Committee on Growth of the National Research Council. The authors are indebted to Mrs. Josephine Tarbet, Miss Evelyn Feaver, Mr. J. Lee Kavanau, and Mr. Allan Schneiderman for technical assistance.

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‡ Present address: Université de Liège, Laboratoire de Chimie Physiologique, Institut Léon Fredéricq, 17 Place Delcour, Liège, Belgium.

1. Chang, S. L., *J. Inf. Dis.*, 1947, v81, 21, 35.
2. Greene, M. R., *J. Bact.*, 1945, v50, 39.
3. Ono, S., *Fukuoka Acta Med.*, 1938, v31, 155.
4. Rosenfeld, W. D., and Greene, M. R., *J. Bact.*, 1941, v42, 165.
5. Savino, E., and Renella, E., *Leptospira y Leptospirosis en la República Argentina*, 1944, Monograph of the Instituto Bacteriológico "Dr. Carlos G. Malbran", Buenos Aires.

organisms.§ Chang(1) and others(2,4,9) have shown that serum is a necessary constituent of the growth medium for many of the leptospira strains, and the presence of this complex substance in test media has considerably limited nutritional studies with these microorganisms. It was the object of the present investigations, therefore, to develop a semisynthetic medium for *Leptospira canicola* which would permit the determination of some of the vitamin and amino acid requirements of this species.

**Experimental.** The media were based on

6. Supniewski, J. W., and Hano, J., *Bull. Internat. Acad. Polon. d. sc. et d. lett., Cl. méd.*, 1937, pages 499-508.
7. Ward, T. G., and Starbuck, E. B., *Proc. Soc. EXP. BIOL. AND MED.*, 1941, v48, 19.
8. Yoshida, N., *Fukuoka Acta Med.*, 1939, v32, 88.
- § Savino and Rennella(5) have reported success in maintaining a *Leptospira icterohemorrhagiae* strain, but not other strains, in a chemically-defined medium consisting of minerals, hematin, asparagine, dextrin and activators (nicotinic acid, nicotinamide, thiamine, pyridoxine, riboflavin, aspartic acid and pimelic acid).

9. Stuart, R. D., *J. Path. and Bact.*, 1946, v58, 343.

Schüffner's medium described by Greene(2) and Rosenfeld and Greene(4). The basal medium was prepared by dissolving the following salts in water and autoclaving and filtering the resulting solution (concentrations given in mg %):  $\text{Na}_2\text{HPO}_4$  (116),  $\text{KH}_2\text{PO}_4$  (154),  $\text{NaCl}$  (468),  $\text{Na}_2\text{CO}_3$  (11.7),  $\text{CaCl}_2$  (11.7), and  $\text{KCl}$  (11.7). 1.6 ml of the basal medium and enough water to make a final volume (including test samples and inoculum) of 7.0 ml were added to each 15 x 127 mm test tube. Heat-labile materials were filter-sterilized and added aseptically after sterilization of the tubes. Heat-stable materials were added before sterilization. The test-tubes were plugged with cotton and sterilized in the autoclave for 30 minutes at 15 lb.

*Leptospira canicola*|| was carried by weekly transfer according to the following procedure. The culture medium (pH 7.2-7.4) was prepared by placing 1.6 ml of basal medium and 4.0 ml of water with 4.68 mg of Witte's peptone in each test-tube. After autoclaving the tubes 0.7 ml of filter-sterilized rabbit serum $\ddagger$  and 0.7 ml of a 7-day culture of *Leptospira canicola* were added aseptically to each tube. The tubes were incubated for 7 days at 32°. A fresh 7-day culture was centrifuged and resuspended in a sterile mixture containing 5.4 ml of water and 1.6 ml of basal medium to prepare an inoculum suspension for use in the nutrition experiments. 0.4 ml or 0.7 ml of freshly prepared inoculum suspension was used to inoculate each tube. After 7 days incubation at 32° the growth in each tube was estimated by measuring the turbidity of the culture with the aid of the Klett-Summerson photoelectric colorimeter. In some of the experiments the turbidity results were verified

|| Strain obtained from Dr. K. F. Meyer, University of California, San Francisco.

$\ddagger$  Pooled blood from decapitated rabbits was stored overnight in a cold room, the serum (containing hemoglobin from partial laking of the red cells) was sterilized by filtration and the sterile filtrate was stored in the refrigerator. The authors are indebted to Mr. Boone of Fontana, California for the rabbits, to Mr. E. A. Murphy and Dr. W. Drell for assistance in collecting the blood, and to the Hyland Laboratories (Los Angeles) for filtering and bottling the serum.

by counting the leptospira in the cultures with the aid of the dark-field microscope.

*Serum fractions.* The basal medium was supplemented with 4.68 mg of Witte's peptone per tube in experiments designed to determine the relative activities of serum and serum fractions in promoting growth of *Leptospira canicola*. It was found that rather extensive dialysis $^{**}$  of the rabbit serum did not appreciably reduce its activity. The response of *Leptospira canicola* to dialyzed rabbit serum in a number of experiments is given in Table I.

Treatment of the dialysed serum with trypsin at pH 8.0 did not reduce the protein content $^{\dagger\dagger}$  or the growth-promoting activity of the preparation. It was assumed that the native proteins of the serum were refractory to trypsin since heated preparations were readily digested. The heated preparations were inactive in promoting growth both before and after digestion with trypsin. Treatment of the dialysed serum with pepsin at pH 3.0 resulted in digestion of the protein and inactivation of the preparation for the test organism. Activity was retained in control samples of dialysed serum held at pH 3.0 under the same conditions. Since the samples were filter-sterilized before testing, it is apparent that the pepsin may have eliminated the essential serum factor either by digesting it or by making it insoluble (a precipitate formed in the pepsin-treated preparation). Fractionation of the dialysed serum by ammonium sulfate precipitation at room temperature followed by dialysis of the fractions to remove the ammonium sulfate resulted in a loss of about 80% of the original activity of the dialysed serum as determined by testing the growth-promoting activity of the combined fractions. The remaining activity did not appear to be concentrated in any one of the fractions which were obtained. It

\*\* 100 ml aliquots of serum were placed in cellulose sausage casings (The Visking Corporation, size 36/32) and suspended in 5 gallon bottles of distilled water in the refrigerator. The distilled water was frequently changed over a 10-day period, and the resulting dialysed serum was filter-sterilized, and kept in sterile bottles in the refrigerator.

$\dagger\dagger$  Tested by precipitation with trichloracetic acid.

NUTRITIONAL STUDIES ON *LEPTOSPIRA CANICOLA*TABLE I. Response of *Leptospira canicola* to Dialysed Rabbit Serum.

Dialysed rabbit serum, ml/tube	Increase in photometer readings after incubation										
	Preparation 1			Preparation 2			Preparation 3		Preparation 4		
	Trial 1	Trial 2	Trial 3				Trial 1	Trial 2	Trial 1	Trial 2	Avg
.14	10	2	3		9		4	6	11	9	6.6
.28	18	9	15		15		12	13	18	14	14
.42	24	22	14		19		21	16	30	23	21
.56	33	26	34		33		30	26	40	28	31
.70	36	39	39		32		37	33	46	36	37

The ml of dialysed serum per tube was calculated as ml of serum before dialysis. Preparations 1 to 4 of dialysed serum were prepared 10/23/48, 7/6/48, 11/12/48, and 12/3/48, respectively. The original serum was collected in March, 1948.

TABLE II. Response of *Leptospira canicola* to Witte's Peptone and to Amino Acid and Vitamin Mixtures.

Additions to media	Increase in photometer readings after incubation					
	Basal medium without peptone			Basal medium with peptone		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
None	15(14-16)	12(11-13)	11(10-11)	32(32-32)	28(28-28)	28(26-30)
Amino acid mixture*	25(24-26)	21(19-25)	26(25-27)	35(34-36)	34(31-37)	36(33-37)
Amino acid + vit. mixture†	32(31-34)	31(29-33)	30(30-31)	37(35-38)	35(33-39)	37(35-39)

The given values are the averages of 2 determinations in the media with no additions and of 5 or 6 determinations in the others. All media contained dialysed serum as described in text. The ranges of the determinations are given in the parentheses.

\* Solution A (described in text) plus glutamine.

† Solutions A and B (described in text) plus glutamine.

seemed likely from the experiments just described that the active factor in the serum was a protein. The loss in activity resulting from ammonium sulfate precipitation was probably due to denaturation of the essential protein.

It is of interest that both the dialysable and non-dialysable fractions of bovine serum contained substances active in maintaining the Reiter strain of *Treponema pallidum* according to the reports of Little and SubbaRow (10) and Whitley and Frazier(11). These investigators have found also that crystalline bovine serum albumin is active for this strain and this finding has been confirmed by Eagle and Steinman(12). Crystalline human and bovine plasma albumins‡ had little activity in replacing the dialysed rabbit serum for

*Leptospira canicola* in the present investigation.

**Peptone requirement.** In the preceding experiments it was found that the basal medium supplemented with Witte's peptone and dialysed rabbit serum supported adequate growth of *Leptospira canicola* (Table I). When Witte's peptone was omitted whole serum, but not dialysed serum (Table II), supported adequate growth. It was apparent, therefore, that essential substances removed from serum by dialysis were supplied by Witte's peptone. The basal medium was supplemented with dialysed serum (in amounts equivalent to 0.7 ml of rabbit serum per tube) in experiments to determine the peptone requirement of *Leptospira canicola*. Trypsin digested casein was found to substitute adequately for Witte's peptone, and in subsequent experiments, 1 ml of the following

10. Little, P. A., and SubbaRow, Y., *J. Immunol.*, 1945, v56, 213.

11. Whitley, H. R., and Frazier, C. N., *Am. J. Syph., Gonor. and Ven. Dis.*, 1948, v32, 43.

12. Eagle, H., and Steinman, H. G., *J. Bact.*, 1948, v56, 163.

‡ The crystalline bovine plasma albumin was a product of Armour Laboratories, Chicago, Ill. The authors are indebted to Dr. E. J. Cohn for a gift of the crystalline human plasma albumin.

amino acid solution per tube, supplemented with 0.7 mg of L-glutamine (heat labile) per tube, was found to substitute partially (Table II) for the peptone (concentrations given in mg %): DL-alanine (51.9), L-arginine monohydrochloride (51.9), L-asparagine monohydrate (5.18), L-cysteine hydrochloride (10.4), glycine (5.18), L-histidine monohydrochloride monohydrate (5.18), DL-isoleucine (51.9), L-leucine (26.0), DL-lysine monohydrochloride (10.4), DL-methionine (5.18), DL-norleucine (10.4), DL-norvaline (10.4), DL-phenylalanine (26.0), L-proline (5.18), DL-serine (10.4), DL-threonine (10.4), DL-tryptophan (5.18), L-tyrosine (10.4), and DL-valine (38.9). The solution just described will be referred to hereafter as Solution A, and unless otherwise indicated the amount used per tube was 1.0 ml.

Growth equivalent to that obtained with peptone (Table II) was obtained with the amino acid mixture (Solution A) when it was further supplemented with 0.7 ml of the following vitamin-purine-pyrimidine-salt solution per tube (concentrations given in micrograms per cent): thiamine hydrochloride (6.7), pyridoxine hydrochloride (10.7), pyridoxal hydrochloride (0.67), pyridoxamine dihydrochloride (0.67), calcium DL-pantothenate (13.4), riboflavin (13.4), niacinamide (13.4), biotin (0.034), *p*-aminobenzoic acid (0.67), folic acid (0.034), choline chloride (51.1), inositol (168), adenine sulfate (80), guanine (80), uracil (80), xanthine (80), MgSO<sub>4</sub> (658), FeSO<sub>4</sub> · 7H<sub>2</sub>O (67), and MnSO<sub>4</sub> · H<sub>2</sub>O

(51.1). The solution just described will be referred to hereafter as Solution B, and unless otherwise indicated the amount used per tube was 0.7 ml.

The basal medium supplemented with dialysed rabbit serum and Witte's peptone was found to support growth of *Leptospira canicola* through a prolonged series of transfers. When the peptone was replaced by Solutions A and B plus glutamine good growth was obtained in the first transfer, but growth was reduced in subsequent transfers. It was apparent, nevertheless, that the semisynthetic medium consisting of the basal medium supplemented with dialysed rabbit serum, glutamine, and Solutions A and B might serve as a basis for determining many of the amino acid and vitamin requirements of *Leptospira canicola*. Experiments to determine these requirements as well as further studies on the nature of the serum factor and the peptone factors required for good growth in serial subcultures are in progress. The results of these experiments are to be given in subsequent papers from the authors' laboratories.

**Summary.** Nutritional experiments with *Leptospira canicola* have been described. A semisynthetic medium containing dialysed rabbit serum, salts, vitamins, amino acids, and purine and pyrimidine bases was developed and appeared to be adequate as a basis for determining amino acid and vitamin requirements of this organism.

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### Respiration of Embryonic Blood. (18148)

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Embryonic respiration has long been a matter of great interest to physiologists. A review of extensive literature on the subject will not be attempted. Hall(1) briefly re-

views contemporary works on the respiratory role of the blood in his report on hemoglobin function in the blood of the chick embryo. These works, however, have been largely qualitative and concerned with the changes in the character and function of hemoglobin

1. Hall, F. G., *J. Physiol.*, 1934, v83, 222.

throughout development. The data here reported are the results of preliminary investigations into the quantitative changes in respiratory rate of the chick embryo, day by day, throughout the period of incubation. Since methylene blue is known to be a catalyst in respiration of adult blood it was deemed feasible to ascertain its effect on respiration of embryonic blood.

*Material and methods.* Determinations of oxygen consumption were made on samples of whole blood. Eggs from white leghorn fowls were incubated in an electric incubator under carefully controlled conditions of temperature ( $100^{\circ}\text{F}$ ) and humidity (65% at  $100^{\circ}\text{F}$ ). Under optimum conditions a hatch in excess of 90% was obtained. Blood samples were taken at ages of 14, 16, 18, 19, 20, 21, 22, 23, 24, 26, 28, and 29 days after the onset of incubation. In the case of the younger embryos the blood was drawn from the vitelline artery into an hypodermic syringe. From those 18 or 19 days of age and older the blood was taken by direct cardiac puncture. Samples 2 cc in volume were used throughout, coagulation being prevented by the addition of 1 mg of heparin. It was found necessary to pool the blood from several embryos to obtain the required volume for a determination, but according to Hall(1), the variation among embryos is so slight as to introduce no significant error in so doing. The apparatus employed was that of Warburg. The vessels used with the apparatus were those having an approximate volume of 20 cc with an "inset-cup" in the center and a side-arm. Brodie solution was used in the manometers. The water bath was maintained at a constant temperature of  $37.7 \pm .05^{\circ}\text{C}$  by an ether and mercury contact thermo-regulator. The manometers were shaken in the bath through an angle of about  $25^{\circ}$  at the rate of 126 oscillations per minute.

In most cases 2 cc of whole blood were placed in the main chamber of the vessel, .5 cc of 10% KOH in the inset cup to absorb the  $\text{CO}_2$  evolved, and .75 cc of .06% methylene blue in .8% NaCl solution in the side arm. In 2 determinations only .25 cc of KOH and .50 cc of methylene blue were

used, the total volume of the contents of the vessel being made up, as always, to 3.25 cc by the addition of .5 cc of .01 M solution of KCN. The manometers were shaken in the water bath for 20 minutes with the stopcocks open to the atmosphere. Equilibration was found to be complete at this time. The stopcocks were then closed and the manometers shaken for 30 minutes before the first reading was made. The manometers were then read at the end of ten-minute periods for, in some cases, one hour, and, in others, one and one-half hours. The curves for samples of the same age followed the same course over periods of either duration. At the end of this time the methylene blue was poured in from the side-arm and readings again made every ten minutes for periods of the same duration as before. Three samples were run concurrently, a fourth manometer containing distilled water in place of the blood was used in every case as a thermobarometer.

Cell volume was determined only in 2 or 3 cases. For obvious reasons it could not be satisfactorily determined at the end of the experiment after methylene blue had been added, and because of the difficulty in obtaining sufficient quantities of blood, it was not

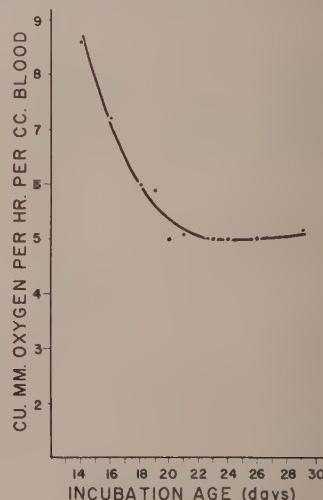


FIG. 1.  
Summary curve showing quantitatively the rate of respiration of embryonic blood during incubation.

TABLE I. Cell Volume and Oxygen Consumption.

Age	Cell volume	O <sub>2</sub> consumption per cc blood per hr
28 days	29.5%	5.1 cu mm
24 "	30.0	5.0
23 "	33.3	5.25
22 "	31.9	5.1
21 "	34.3	5.1
20 "	30.8	5.0
19 "	34.9	5.9
18 "	32.1	6.0
—		19.0-35.7*
—		

\* Figures of Tipton<sup>2</sup> for chicken blood.

investigated at the beginning. Tipton<sup>(2)</sup> demonstrated an apparent lack of correlation between red cell count, red cell size and red cell volume, on the one hand, and oxygen consumption, on the other, in the blood of the adult chicken. Cell counts were not made in this experiment but cell volume determinations made on the blood of embryos, by means of the Wintrobe<sup>(3)</sup> hematocrit tube, showed a lack of correlation with rate of oxygen consumption similar to that found by Tipton<sup>(2)</sup>.

*Results.* The relative rate of respiration of the embryonic blood of the chick was found to decrease steadily from 14 to 19 days after the onset of incubation. From this time onward until 29 days after the onset of incubation the rate is virtually constant (Fig. 1). Cell volume at various ages was determined by means of the Wintrobe hematocrit. The percentage cell volume was found to fluctuate within rather wide limits although, in general, it becomes less as the age increases. However, there seems to be no direct correlation between cell volume and the rate of respiration in embryonic blood (Table I).

Barron and Harrop<sup>(4)</sup> and Tipton<sup>(2)</sup> have previously pointed out that methylene blue has no appreciable effect on the respiration of chick erythrocytes. (These findings are here confirmed.) Although this is true, methylene blue does demonstrate its usual capacity to neutralize the effect of cyanides. In 2 determinations .5 cc of a .01 M solution of

KCN was added to the usual sample of blood. This was not a sufficient quantity to completely suppress respiratory activity but it did show a marked inhibitory effect on it (Table II).

Upon the addition of methylene blue to blood containing cyanides it acts only to overcome the effect of the cyanide. The rate of respiration of the sample returns toward that found for normal unadulterated blood or blood containing methylene blue but no cyanide. Thus, although methylene blue shows no effect on normal respiration it does neutralize the effects of KCN and raises the oxygen consumption close to but not above normal at 29 days. At 20 days the effects of KCN are neutralized but the recovery in oxygen consumption was found to be slight. Reasons for this differing effect with age have not been established.

*Discussion.* The respiratory rate of embryonic blood of the chick, determined by the oxygen consumption of samples of a given volume of blood, is found to decrease gradually from 14 to about 19 days after the beginning of incubation. From this time onward there appears to be little change. Hall<sup>(1)</sup> postulates a change in the character of the hemoglobin molecule from the embryonic to the adult condition. When the embryo consumes only the oxygen that can diffuse through the egg shell it is respiring from an atmosphere of low oxygen tension. When this condition prevails, a hemoglobin with a high affinity for oxygen is needed. When the egg shell becomes permeable to gases, as it does with age, and later when the chick tears the shell membrane and breathes from the air-space and eventually from the atmosphere directly, the oxygen tension of its atmosphere is greater and a hemoglobin with a somewhat lesser affinity for oxygen is supplied. It is the changing ratio of the quantities of these types of hemoglobin that accounts for the shift of the dissociation curve for embryonic blood of the chick as age progresses so that the position of the adult dissociation curve is gradually approximated (Hall)<sup>(1)</sup>.

It is during the earlier stages of development, when the blood volume and the oxygen

2. Tipton, S. R., *J. Cell. and Comp. Physiol.*, 1933, v3, 313.

3. Wintrobe, M. M., *J. Lab. and Clin. Med.*, 1929, v15, 287.

4. Barron, E. S. G., and Harrop, G. A., Jr., *J. Biol. Chem.*, 1928, v79, 65.

TABLE II. Effect of KCN and Methylene Blue on Oxygen Consumption, Age, O<sub>2</sub> Consumption per cc Blood per Hour.

Age	Normal blood	Blood plus KCN and M.B.	Blood plus KCN	Increase with KCN and M.B. over KCN without M.B.
20 days	5.0 cu mm	1.4 cu mm	1.2 cu mm	.20 cu mm
29 "	5.2	5.0	2.2	2.80

tension are low; that hemoglobin with the highest affinity for oxygen is present. At this stage a given volume of blood shows a high oxygen uptake. In later stages, when the blood volume and oxygen tension are greater, there is present a greater number of molecules of hemoglobin having a lesser affinity for oxygen. At this stage the same volume of blood has a smaller oxygen uptake. However, this is only true if the percentage cell volume and the needs of the embryo are considered. As pointed out above, there seems to be no very exact correlation between cell volume and rate of oxygen consumption of blood (Table I).

The growth rate from 14 days of incubation is very small and steadily becomes less. This seems to indicate that the needs of the embryo are becoming relatively constant during this period.

Previous workers [Wright(5) and Tipton (2)] have demonstrated the fact that young blood cells produced during anemia have a much higher respiratory rate than have mature red blood cells. These "primitive" cells, however, are formed in a different locale than are the young cells found in embryonic blood. Thus, though the two be structurally similar, they may be physiologically dissimilar. Primitive cells produced during anemia are much larger than mature red blood cells (Tipton) (2). These may show their high respiratory rate because of the fact that their production is initiated in a previously established hematopoietic organ by a complex mechanism set in motion by the lack of a sufficient quantity of cells. Thus, their high respiratory rate may be due to a process of rebuilding or differentiation.

Young embryonic cells, on the other hand,

5. Wright, G. Payling, *J. Gen. Physiol.*, 1930, v14, 179.

arising from the generalized mesenchyme, because of their rapid growth, would be expected to have a high metabolism and therefore their oxygen uptake should be greater, as shown.

The growth rate of the embryo is ever decreasing from at least 7 days of incubation onward. Since fewer and fewer young cells are being added to the circulation, the ratio of mature or maturing red blood cells to young or primitive red blood cells would seem to be going toward the side favoring the older cells. If then the respiratory rate of younger cells is greater than that of the mature cells it would seem that the gradual decrease in the rate of respiration of embryonic blood found to take place from 14 to 19 days of incubation might conceivably be due in part to the fact that there is a gradual diminution in the number of young red blood cells in comparison with the number of mature cells in the circulation. Supporting this, to bring about the drop in respiratory rate with age, there might also be considered the change in the relative quantities of the two types of hemoglobin present in the developing and mature chick, as postulated by Hall(1).

*Summary.* 1. The rate of respiration of a given volume of embryonic blood gradually decreases from the 14th to the 20th day of incubation. From this time until 29 days after the onset of incubation (the greatest age investigated) it remains virtually constant. 2. It is suggested that the decrease in respiratory rate is due to a decrease in the number of young cells having a high respiratory rate and a hemoglobin with a lesser affinity for oxygen. 3. Methylene blue shows no catalytic effect on the rate of respiration of embryonic blood of the chick. It will, however, counteract the inhibitory effect of KCN when present.

## Phosphorylation of Ribose and Adenosine in Yeast Extracts.\* (18149)

HENRY Z. SABLE,† (Introduced by Carl F. Cori.)

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Yeast extracts are known to ferment ribose and ribose-5-phosphate, the latter at a rate comparable to that of glucose(1). Intact yeast cells can convert added adenosine to adenosine triphosphate (ATP) when inorganic phosphate and a fermentable substrate are also present(2). In the course of an investigation of the metabolism of pentose derivatives in yeast extracts, enzymes have been found which can bring about the phosphorylation of ribose and of adenosine, when ATP is added.

**Experimental.** Brewers' yeast and bakers' yeast were both supplied by Anheuser-Busch, Inc. The brewers' yeast was collected from a vat at the end of fermentation, and pressed. Upon arrival in the laboratory it was washed and dried according to Lebedev(3) and was stored at 2°C. Maceration juice was prepared by autolysis at 37°C, also according to Lebedev's original instructions. The bakers' yeast was washed several times with tap water and distilled water, and dried at a temperature not exceeding 15°C. Maceration juice was prepared from bakers' yeast by stirring mechanically at 2°C a mixture of 500 g dry yeast, 1150 ml water and 75 ml toluene, over periods of 5 to 7 days. In all cases, solids were removed by high speed centrifugation on a Servall angle-head centrifuge. The reaction mixtures contained, in a final volume

of 5.5 ml, 10 to 15 micromoles of the sugar to be tested (or adenosine), 10 to 15 micro-moles of ATP, and 0.6 to 0.8 ml of the enzyme solution. All incubations contained 0.006 M NaF and 0.01 M MgCl<sub>2</sub>, although the need for Mg<sup>++</sup> has not been definitely established. The reaction mixtures were buffered at pH 7.6 to 8.0 in various experiments, with tris(hydroxymethyl)aminomethane(4), final concentration 0.025 M. The amine was purified as described elsewhere(5). When changes in phosphorus were to be measured, aliquots were deproteinized by addition of an equal volume of 6% perchloric acid and the filtrates chilled in ice until analyzed. Phosphorus was determined by King's modification of the Fiske and Subbarow method(6), before and after 10 minutes hydrolysis in 1 N H<sub>2</sub>SO<sub>4</sub> at 100°. When sugar was to be measured, aliquots were deproteinized with Ba(OH)<sub>2</sub> and ZnSO<sub>4</sub>(7). In this method phosphorylated compounds are adsorbed by the solid phase. Reducing power of the filtrates was measured by the method of Nelson(8).

Evidence for a ribose phosphorylating enzyme is presented in Fig. 1. Curves A, B, and C represent the amount of free ribose actually present in the filtrates. Curve D represents the utilization of ATP, as measured by the disappearance of acid-labile phosphate. The values in curve D are the differences between two parallel reaction mixtures, one with and one without ribose added. Since one deals with relatively small differences between large numbers, this type of determination is less accurate than direct determination of sugar. The first sample in each case was taken at 0.5 minute. The enzyme solu-

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† Postdoctorate Fellow of the National Institutes of Health, United States Public Health Service, 1948-50.

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## RIBOKINASE AND ADENOSINE PHOSPHOKINASE

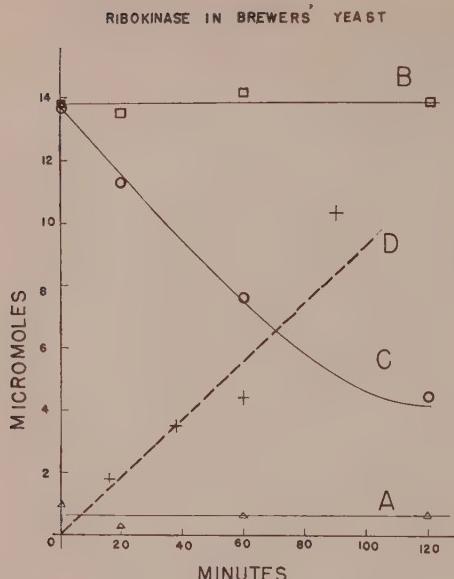


FIG. 1.

Ribokinase in a fraction obtained from brewers' yeast. Curves A, B, and C show utilization of ribose: A—ATP present, ribose omitted; B—ribose present, ATP omitted; C—both ribose and ATP present. Curve D represents utilization of ATP. Details in the text.

#### ADENOSINE PHOSPHOKINASE IN BREWERS' YEAST

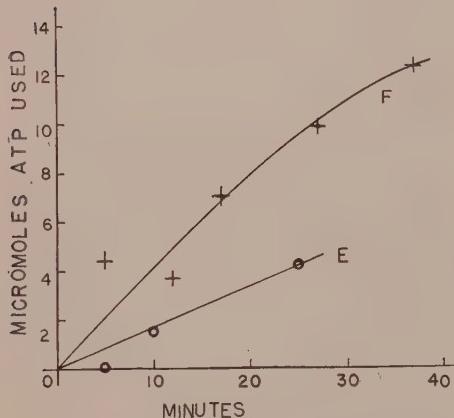


FIG. 2.

Adenosine phosphokinase in brewers' yeast. The data are obtained as in the case of Curve D, Fig. 1, and represent utilization of ATP when adenosine is added. Curve E: Lebedev juice, 18 mg protein added. Curve F: .45-.65 saturated ammonium sulfate fraction, 24.9 mg protein added.

tion in this case was a protein fraction precipitated from brewers' yeast maceration juice with ammonium sulfate, between 0.45 and 0.65 saturation, at pH 7.6. The same protein solution failed to affect D(+)xylose, D(-)arabinose, L(+)arabinose, and D-2-desoxyribose. Because of this specificity the enzyme is designated *ribokinase*. The same extract did phosphorylate glucose at a rapid rate, and also adenosine (Fig. 2). Since a fivefold concentrate of ribokinase from bakers' yeast did not phosphorylate adenosine, it was concluded that a separate enzyme is involved, which is designated as *adenosine phosphokinase*.

Partial purification of ribokinase has been carried out, starting with maceration fluid obtained from bakers' yeast. It was established first that crystalline yeast hexokinase (9) is unable to phosphorylate either ribose or adenosine. Table I shows that at a very early stage one obtains a fraction which is rich in hexokinase and devoid of ribokinase, and another fraction which is relatively enriched in ribokinase and poor in hexokinase. This is conclusive proof that different enzymes are involved. The steps in the purification are as follows:

All operations except the assay are carried out in a cold room at 2°C. The autolysate, pH 6.1, is adjusted to pH 7.5 to 7.6 with dilute ammonium hydroxide. A solution of ammonium sulfate, pH 7.6 and saturated at room temperature is added, 94.3 ml being added per 100 ml autolysate. The resulting solution is 0.485 saturated in ammonium sulfate; it is chilled in ice for  $\frac{1}{2}$  hour, and the precipitate which has formed is separated by high speed centrifugation and discarded. The ammonium sulfate concentration is then raised to 0.625 saturation by adding 38.7 ml of the ammonium sulfate solution to each 100 ml of the supernatant solution. The mixture is again chilled in ice for  $\frac{1}{2}$  hour and centrifuged. The supernatant fluid is discarded. The precipitated proteins are dis-

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TABLE I. Separation of Ribokinase and Hexokinase in Autolyzed Bakers' Yeast.

Fraction	Total protein, g	Ribokinase*		Hexokinase† Apparent purity, %
		Units/mg	Total units	
(1) 141 hr autolysate	45.8	0.89	41000	1.87
(2) Ammonium sulfate fraction, .485-.625 saturation	16.0	1.45	23100	2.34
(3) Pte. at pH 4.6 ionic strength .02	2.6	4.75	12250	0.32
(4) Supernatant fluid of (3)	13.4	Not detectable		4.63‡

\* A unit of ribokinase is the amt. of enzyme that would catalyze the phosphorylation of .10 micromol of ribose, at the initial rate observed.

† Hexokinase was measured under optimal conditions(9). The initial rate of the reaction was determined and the number of  $\mu$ g of hexokinase calculated, using the turnover number of 32  $\mu$ g of glucose phosphorylated per  $\mu$ g of hexokinase per min. The purity of the hexokinase was then calculated from the amt of protein(10) used in the reaction.

‡ The apparent gain in total hexokinase in fraction(4) may be due to the removal of interfering enzymes by the acid precipitation.

solved in the minimum quantity of cold distilled water, and dialysed for 12 to 14 hours against two changes of acetate buffer, pH 6.0, ionic strength 0.05. The volume of buffer should be 200 to 500 times as great as the volume of protein solution. The protein solution is then dialysed for an additional 6 to 8 hours against acetate buffer pH 4.5 to 4.6, ionic strength 0.02. A precipitate begins to form inside the dialysis sac shortly after dialysis against the more acid buffer is begun, and increases in amount. At the end of this dialysis the precipitated protein is collected by centrifugation. The precipitate dissolves with difficulty. It has been found most convenient to suspend the precipitate in a small amount of cold water, adjust the pH to 6.0 with a concentrated solution of sodium acetate, and then add a concentrated solution of sodium chloride, with stirring, until the precipitate dissolves. The final solution is about 0.3 M in salts. Occasionally this precipitate contains some irreversibly denatured protein, which does not dissolve. The protein solution is then adjusted accurately to pH 6.0, ionic strength 0.30, by dialysis against acetate buffer of this concentration, the buffer being changed once in the course of an 8-hour dialysis. The protein solution may now be used for fractionation with alcohol. A solution 80% (by volume) of ethanol in water, ionic strength 0.02 (NaCl) has been used in the present work. The protein solution is chilled in an ice-salt bath and 0.33 volume of the

alcohol solution added, the temperature being maintained between -2° and -5°C. The solution is then kept at -2° for 5 to 10 minutes, and centrifuged in glass cups, during which time the temperature rises to +1.5°. The supernatant fluid is discarded. The precipitate is dissolved by adding 0.5 volumes of concentrated acetate buffer, pH 6, chilled to -5° or lower. The excess alcohol and salts are then removed by dialysis for 4 or 5 hours against a dilute buffer of pH 6.0. A precipitate may form on dialysis. This has never been found to contain any of the desired activity. The final solution is usually a bright yellow.

The redissolved precipitate, obtained after dialysis against acid, retains its potency over a period of months in a freezer box at -20°C. The fraction obtained by alcohol treatment does not have such good storage properties. In several experiments, the purification achieved by alcohol treatment in this way has varied between two- and threefold over the previous step. This represents, therefore, a total purification of ten- to fifteenfold over the maceration juice.

In the process of purification, certain interfering enzymes accompany ribokinase. The most highly purified solutions still contain very active phosphatases and the systems that degrade ribose-5-phosphate anaerobically. For this reason, it has not been possible to isolate and to identify the product of the reaction. Paper chromatography of phosphate esters has been carried out successfully by

Hanes and Isherwood(11) and by Benson *et al.*(12). An attempt was made to identify the phosphorylated compound which accumulates during the ribokinase reaction. The reaction was carried out on a scale five times larger than usual; as controls, mixtures were incubated in which ATP was added and ribose omitted, or ribose added and ATP omitted. An additional incubation mixture contained the enzyme solution and authentic ribose-5-phosphate, with no ATP or ribose added. At the end of the incubation period the mixtures were deproteinized with perchloric acid, and the filtrates neutralized. Barium acetate was added in excess, and finally ethanol was added to bring the final concentration to 80%. The precipitated barium salts were collected and dried, resuspended in water and barium removed with sulfuric acid. The supernatant fluids were neutralized with sodium hydroxide and evaporated to small volume at room temperature, and the resultant solutions used for chromatography. The results are summarized in Table II. From the  $R_f$  values found it was

TABLE II. Chromatography of Sugars on Filter Paper Strips.

Whatman No. 4 paper was washed with 2N acetic acid and then with 8-hydroxyquinoline in 50% ethanol(11).

Solvent: 25 ml  $H_2O$  + 22.5 ml propionic acid + 52.5 ml tert.-butanol, prepared just before use.

Temp.: 29-30°C.

Material	$R_f$
A. Authentic substances	
Ribose-5-phosphate	.22
L-ketoxylose-1-phosphate	.21
Fructose-6-phosphate	.18
Fructose 1,6-diphosphate	.095
Ribose	.50
B. Incubated materials	
Ribose + ATP	.097
" alone	None found
ATP alone	" "
Ribose-5-phosphate	.14

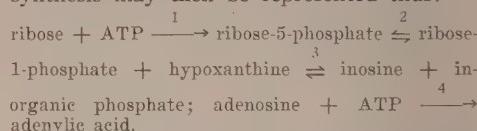
The sugars were detected by treating the dried paper with the Folin-Wu sugar reagents, heating for 5 min. at 110°C after applying the alkaline copper solution, and then treating with phosphomolybdic acid(13).

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suspected that fructose-6-phosphate or fructose-1, 6-diphosphate were implicated. However, these substances could not be detected in enzymatic tests, using purified enzymes (14-16). Thus the nature of the product is still undecided.

*Discussion.* The ribokinase reaction (reaction 1) may be one of the important steps in nucleic acid synthesis in yeast, particularly if the product will be found to be ribose-5-phosphate. Some of the early steps in the synthesis may then be represented thus:



An enzyme catalyzing reaction 2 and designated as *phosphoribomutase* has actually been demonstrated in mammalian tissues(17). This was shown by adding ribose-5-phosphate and hypoxanthine to extracts of rabbit muscle and rat liver, and demonstrating the formation of inosine(18). The existence of phosphoribomutase might have been inferred from the products formed in the anaerobic metabolism of nucleosides and nucleotides (19,20). The yeast extracts used in these studies also contained a potent nucleoside phosphorylase, catalyzing reaction 3. Reaction 1 has not yet been demonstrated in mammalian tissues. Youngburg(21) reported that homogenized kidney cortex could not phosphorylate ribose, and in the present investigation it has been found that a water extract of rat muscle, which contains an

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active glucokinase, is unable to phosphorylate ribose. While reaction 4 probably represents a step in the synthesis of nucleic acid or of additional ATP from adenosine by yeast, mammalian tissues have not yet been found to contain a nucleoside phosphokinase. The extract of rat muscle was tested with adenosine with negative results. Homogenized kidney cortex, prepared as described by Colowick *et al.*(22) was tested with adenosine and guanosine. Nucleoside and inorganic phosphate disappeared and acid labile phosphate accumulated, under aerobic conditions. However, no nucleotides accumulated, and the acid labile phosphate was found to be inorganic pyrophosphate(23). Rapport *et al.*

22. Colowick, S. P., Kalckar, H. M., and Cori, C. F., *J. Biol. Chem.*, 1941, v137, 343.

(24) have reported similar findings.

*Summary.* Yeast autolysates have been found to contain enzymes which catalyze the phosphorylation of ribose and of adenosine, when ATP is added as a phosphate donor. The enzymes, designated ribokinase and adenosine phosphokinase, have been partially purified, but the best preparations still contain large amounts of interfering enzymes, making it impossible to isolate and to identify the products of the reactions. The possible physiological significance of the reactions is discussed.

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Received July 31, 1950. P.S.E.B.M., 1950, v75.

## Protein Digestibility and Trypsin Inhibitor Activity of Legume Seeds. (18150)

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The poor nutritional quality of some crude legumes has been attributed in early studies to low digestibility(1) and it has been shown that *in vitro* digestibility of crude beans is lower than that of cooked beans(2). Later, factors have been found in a number of crude legumes which inhibit the *in vitro* activity of trypsin(3). However, a purified trypsin inhibitor from soy beans did not inhibit growth of rats and chicks(4). Moreover, no correlation between improvement of growth promoting action of legumes after autoclaving and their trypsin inhibitor content was found in a recent study(5). Some legumes contain

heat labile factors which inhibit growth and are probably not identical with trypsin inhibitors(6). No correlation could be detected between growth depression by crude legume seeds and their trypsin inhibitor activity(7). It therefore seemed to be of interest to study the *in vivo* digestibility of some legumes in the crude and autoclaved form and to compare them with their activity to inhibit the action of trypsin *in vitro*.

*Experimental.* The protein digestibility experiments were made with young Sprague-Dawley rats of about 100 g of weight. For each experiment 2 male and 2 female rats were housed in single screen bottomed cages. Food and water were given *ad libitum*. Food consumption was determined and feces were collected every 3 days, dried at 70°C, weighed, and analysed for N. With the mentioned exceptions, the diets were composed mainly of starch and the amount of the dried and

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TABLE I. True Digestibility of Proteins of Some Crude and Autoclaved Legumes in Growing Rats and Their Trypsin Inhibitor Activity.

Legume	Scientific name	Digestibility		Trypsin inhibitor $10^{-4}$ units/g
		Crude	Autoclaved	
Black kidney beans	<i>Phaseolus vulgaris</i>	64.0	76.5	3.90
Red kidney beans	,,	56.0	79.5	4.25
Hyacinth beans	<i>Dolichos Lablab</i>	56.5	81.6	4.38
Soy beans	<i>Glicinso soya</i>	70.1	85.4	4.15
Lima beans	<i>Phaseolus lunatus</i>	34.0	51.3	4.04
Pigeon peas	<i>Cajanus indicus</i>	59.1	59.9	2.77
Cow peas	<i>Vigna sinensis</i>	79.0	82.6	1.91
Lentils	<i>Lenis esculenta</i>	88.3	92.6	1.78

ground seeds to contain 10% of crude protein ( $Nx 6.25$ ); the composition of these diets and the treatment of the legume seeds was the same as described earlier(8). The crude kidney beans and hyacinth beans were assayed as a mixture of 20% of the seeds with 80% of a commercial rat diet of known digestibility. This technic, although less accurate, had to be adopted as these seeds are toxic for rats and food consumption was very low when they were administered in the same diet as the other legumes studied. In a separate experiment, the protein digestibility of cooked kidney beans was determined using both experimental diets; the results differed only by 0.8%. To compute the true digestibility from the values of apparent digestibility, the endogenous nitrogen excretion in the feces of the strain of rats used was determined separately and found to correspond to an excretion of 1.25% of crude protein. This value was used for all the calculations. The digestibility of each sample was determined from 3-8 times with 1-4 groups of rats. The results given in Table I are the averages of these experiments. Trypsin inhibitor activity was determined according to the technic of Borchers and Ackerson(3).

Most of the legume samples were obtained from the Genetical Division of the Ministry of Agriculture. The samples of hyacinth beans and of lima beans were purchased in Barquisimeto and are local varieties. The lentils were bought at the local market from an imported lot.

**Results.** The results are summarized in

Table I. Only in kidney beans, soy beans, lima beans and hyacinth beans, a significant difference in the protein digestibility between the crude and autoclaved legumes could be detected. These samples had trypsin inhibitor activities of more than  $3 \times 10^{-4}$  units/g. All the other samples studied were less active in inhibiting trypsin action *in vitro* and the proteins of these seeds were nearly equally well digested whether given crude or autoclaved. Waterman and Johns(2) found a difference of the *in vitro* digestibility between raw and autoclaved kidney beans of about 15% which is in fair agreement with the *in vivo* values found in this study.

The digestibility of the lima bean sample was surprisingly low. The pigeon peas had also an unusual low digestibility coefficient; we have described recently the great variability found in different varieties of this legume in respect to the digestibility of its proteins(9). The sample of lentils studied was fairly active in inhibiting the action of trypsin *in vitro* while Borchers and Ackerson (3) found this legume to be inactive in this respect.

**Summary.** Protein digestibility of raw and autoclaved legume seeds were determined in growing rats and the trypsin inhibitor content of the same seeds was measured *in vitro*. The proteins of kidney beans, soy beans, lima beans, and hyacinth beans were 12-25% more completely digested when autoclaved as compared with the raw seeds. These samples had also the highest trypsin inhibitor activities.

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Serum and Tissue Glycoproteins in Mice Bearing Transplantable Tumors.\*  
(18151)

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It has long been known that protein-bound carbohydrate increases in the blood of cancer patients(1). More recently, Seibert *et al.* (2) observed a rise in serum polysaccharide and in  $\alpha_2$  globulin in tuberculosis, sarcoidosis and carcinoma; they attributed these increases to tissue destruction. Winzler *et al.*(3) and Winzler and Smyth(4) found an increase in the mucoprotein fraction of cancer and pneumonia sera, which they attributed to an abnormality in protein metabolism. Shetlar *et al.*(5) studied a serum polysaccharide component which increased following inflammation. They postulated that this rise was associated with tissue proliferation and repair. Recent studies(6) suggested that increased circulating sugar-containing proteins might arise from a connective tissue component or components of a glycoprotein nature. Such components may be visualized histochemically by the McManus-Hotchkiss periodic acid leucofuchsin technic, and can be demonstrated normally to be water and alcohol insoluble. Following traumatic injury to tissue and also after induced lung edema, the local state of the ground substance of the connective tissue changed, and it became water soluble. This change was ascribed to the secretion of de-

polymerizing enzymes which produced breakdown of a highly polymerized, insoluble ground substance to less highly polymerized residues. It was postulated that the latter might be soluble and diffusible, and appear as glycoprotein components of serum. Part of the evidence for the above thesis rested on the finding, in the region of fast growing tumors, of jelly-like, easily ruptured connective tissue strands, in place of the coherent, resistant connective tissue normally encountered, and on the demonstration of water solubility of the ground substance of this tissue in histological sections.

*Materials and methods.* Young adult Swiss and C<sub>3</sub>H male mice were used. They received implants, into the abdominal subcutaneous region, of the fibrosarcomas: Earle HGW, OA and L (obtained through the courtesy of Dr. Thelma Dunn, National Institute of Cancer). After varying lengths of time, small (2-3 mm), large (1.0 cm), and occasionally very large (over 2.0 cm) tumors were found. Usually 4 or more animals were taken in groups of comparable tumor size, anesthetized, and bled from the right heart. Connective tissue from the tumor site was collected by means of fine scissors and forceps, transferred immediately to a chilled tube and the pooled material lyophilized. Blood and subcutaneous connective tissue were also obtained from groups of normal mice; in these, the skin was pinned out and connective tissue stripped off by fine toothed forceps. For determining plasma glycoproteins the method of Winzler *et al.*(3) was used.<sup>†</sup> Plasma was diluted 1:1 with water and plasma proteins precipitated by the addition of 2 volumes of 0.75M perchloric acid; from the filtrate, glycoproteins were precipitated with  $\frac{1}{2}$  volume of 5% phosphotungstic acid in 2N HCl. Gly-

\* Supported by a grant from the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

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<sup>†</sup> We are greatly indebted to Dr. R. J. Winzler for supplying details of their methods antecedent to publication.

## GLYCOPROTEINS IN TUMOR BEARING MICE

TABLE I. Plasma Mucoproteins in Normal Mice and Mice Bearing Transplantable Tumors.

	No. of animals	Sugar as galactose-mannose, mg/100 cc	Tyrosine, mg/100 cc	Sugar/tyrosine
Normal Swiss	40	16.9 ± 1.95*	3.96 ± 0.6*	4.05
Tumor bearing Swiss				
Earle HGW—large	5	28.0	7.35	3.80
medium	3	18.4	6.15	3.00
small	16	16.4	4.30	3.85
Normal C <sub>3</sub> H	6	17.7	3.8	4.65
Tumor bearing C <sub>3</sub> H				
Earle HGW—large	5	63.5	5.78	11.0
OA—large	6	57.6	5.45	10.6
L—large	8	34.7	7.05	4.9
L—small	5	19.0	5.4	3.5

\* Stand. error of mean.

TABLE II. Mucoproteins of Subcutaneous Connective Tissues of Normal Mice and of Connective Tissue Associated with Subcutaneously Transplanted Tumors.

	No. of animals	Sugar as galactose-mannose, mg/100 g	Tyrosine, mg/100 g	Sugar/tyrosine
Normal Swiss	50	230 ± 37.5*	26.6 ± 4.5*	8.6
Tumor bearing Swiss				
Earle HGW—large	5	300	50.0	6.0
medium	3	275	55.0	5.0
Normal C <sub>3</sub> H	6	181	19.2	9.5
Tumor bearing C <sub>3</sub> H				
Earle HGW	5	565	62.5	9.0
OA	6	—	29.5	—
L—large	8	305	23.5	12.8
L—small	5	392	28.5	13.8
L—very large	1	400	92.0	4.4

\* Stand. error of mean.

coprotein was estimated by the use of two methods: (1) sugar with the orcinol reagent, against a galactose-mannose standard; (2) protein with the phenol reagent of Folin and Ciocalteu, against a standard of tyrosine.

The dried connective tissue samples were ground with methanol-chloroform (1:1) in a small mortar. The solvent was removed by centrifugation and replaced successively by acetone and ether. Finally the defatted residue was incubated overnight with absolute alcohol to effect denaturation. The alcohol was removed and the connective tissue dried and weighed. It was triturated with a small quantity of phosphate buffer (1.0-2.0 ml) at pH 7.0 for 30 minutes, after which the tissue fragments were removed by brief centrifugation at 18000 G. The supernatant solution was removed with a syringe and thereafter treated similarly to the sample of diluted blood plasma in the Winzler procedure.

**Results.** Enhanced amounts of plasma glycoproteins were readily shown in the presence of large tumors. With practical uniformity, values for glycoproteins whether determined as carbohydrate or as protein were increased in both strains of mice and for all tumors studied (Table I). The individual sugar/tyrosine ratios were somewhat variable, but the means (normal, 4.2; tumor, 4.1) essentially agree with the values found in the human by Winzler *et al.*(3) (normal,  $3.69 \pm 0.23$ ; cancer,  $3.85 \pm 0.30$ ). Partly these variations may be explained by difficulties of blood collection and determination of small samples. In two series, the plasma glycoprotein values were relatively little increased for small and medium sized tumors and greatly increased for very large tumors. That agreement may not always be absolute between tumor size and carbohydrate release may be due to the unpredictable biological

status of any tumor of a given size.

Water soluble glycoproteins of tumor connective tissue show distinct increases as compared with normal connective tissue in Swiss and C<sub>3</sub>H mice implanted with various tumors (Table II). Both sugar and protein (tyrosine) values are elevated. In some cases the sugar/tyrosine ratios show wide fluctuations from the expected values; the reason for this has not been explained.

*Summary and conclusions.* Serum glycoproteins are increased in mice bearing trans-

plantable tumors. Water soluble glycoproteins are increased in the connective tissue bordering and abutting on the tumor. These findings support the thesis that increased circulating glycoproteins arise from the ground substance of the connective tissue at the site of invasive growth by a process of depolymerization, whereby smaller, water soluble and diffusible glycoprotein moieties are produced.

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### Quantitative Estimation of Plasma Accelerator-Globulin. (18152)

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In a previous publication(1), a method was described whereby oxalated bovine plasma could be rendered free from Ac-globulin. This solution (HgCl<sub>2</sub>-plasma), containing 50-60 Iowa units/ml of prothrombin, has provided a stable, reliable source of prothrombin for the quantitative estimation of Ac-globulin in plasma. The method of assay, based upon the use of three variables—conversion rate, thrombin yield, and onset of thrombin formation, is a modification of the method of Ware and Seegers(2).

*Method.* 1. Human and dog whole blood is collected in 1.85% potassium oxalate; 7 parts of blood to 1 part of anticoagulant are used. For rats, 1 ml of blood is taken into a syringe containing 0.23 ml of 1.85% potassium oxalate. The blood is centrifuged at 5000 r.p.m. for 15 minutes, and the hematocrit and oxalate correction factor recorded.

2. Determine the amount of prothrombin in the plasma to be tested for Ac-globulin using the modified two-stage technic(3,4).

1. Carter, J. R., and Warner, E. D., Proc. Soc. EXP. BIOL. AND MED., 1950, v74, 30.

2. Ware, A. G., and Seegers, W. H., *J. Biol. Chem.*, 1948, v172, 699.

3. Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, v114, 667.

4. Ware, A. G., and Seegers, W. H., *Am. J. Clin. Path.*, 1949, v19, 471.

3. Into a chemically clean glass test tube (120 x 13 mm), place 4 ml of physiologic saline.

4. Add HgCl<sub>2</sub>-plasma in an amount such that the prothrombin in the HgCl<sub>2</sub>-plasma, plus the amount of prothrombin in the plasma to be assayed for Ac-globulin equals 50 units.

5. To the diluted HgCl<sub>2</sub>-plasma, add 0.08 ml of the human plasma (0.008 ml for dog, rat, and beef plasma) to be assayed.

6. Add physiologic saline to the tube to bring the total volume to 5 ml. The prothrombin concentration is now 10 units/ml. Swirl tube to insure thorough mixing of components.

7. To 1.5 ml of incubation mixture,\* add 0.5 ml of contents of the dilution tube (step 6).

8. Incubate at 28°C for 5, 8, 11, 14, 17, 23, 26, and 30 minutes. At the end of each of these time periods, add 0.2 ml of the contents of the incubation tube (step 7) to 0.05 ml of a 1.0% solution of Armour bovine fibrinogen, and record the clotting time.

\* Incubation mixture is prepared as follows: calcium saline (1% CaCl<sub>2</sub> in physiologic saline), 3 parts; 15% acacia (containing 0.04% calcium by weight), 2 parts; imidazole buffer (pH 7.2), 1 part. To 10 ml of this solution is added 5 ml of heated crude lung thromboplastin.

## ESTIMATION OF PLASMA AC-GLOBULIN

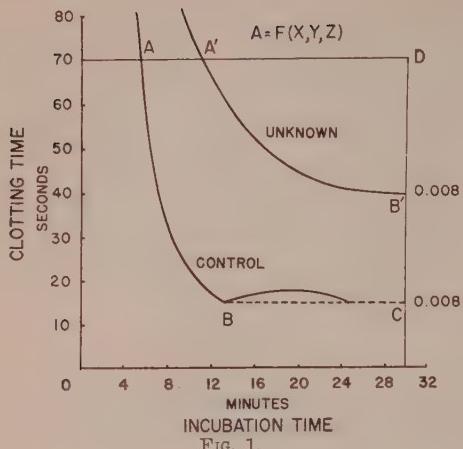
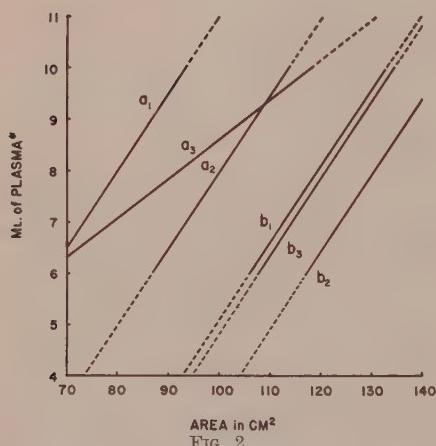


FIG. 1.



\* ml of plasma  $\times 10^{-2}$  human;  $\times 10^{-3}$  dog, rat.  
 a—undefibrinated plasma;  
 b—defibrinated plasma;  
 l—human, 2—dog, 3—rat. Throughout this paper,  
 defibrinated plasma is that which has been treated  
 with 10 units/ml of thrombin and the fibrin clot  
 removed after standing 20 min.

9. On arithmetic graph paper (Fig. 1), plot the activation curve: clotting time in seconds (ordinate) against the incubation time in minutes (abscissa). Draw a vertical straight line through the 30 minute incubation time, and a horizontal straight line through the 70 second clotting time. From the point of maximal thrombin yield on the activation curve (B), draw a straight horizontal line to the 30 minute vertical line.

10. Measure the area in  $\text{cm}^2$ , preferably with a planimeter, bounded by the activation

curve, and the horizontal and vertical straight lines as set forth above (area A, B, C, D). The area derived from the plasma to be assayed for Ac-globulin activity and that derived from the same volume of a normal control plasma is measured.

11. By referring the areas to a standard reference curve (Fig. 2), the Ac-globulin activity can be expressed in percent of normal. Through a point determined by the volume of normal control plasma used in the assay and its corresponding derived area, draw a line parallel to those on the reference curve. Using this line, mark the point on it at which the area obtained with the unknown plasma crosses it. The corresponding volume of plasma (ordinate) is therefore that amount which contains Ac-globulin activity equivalent to that which would have been obtained with the normal control plasma. Therefore:

$$\text{derived ml of unknown plasma} \times \text{ocf} \dagger$$

$$\frac{\text{actual ml of plasma used} \times \text{ocf of normal control}}{\times 100} = \% \text{ Ac-globulin activity.}$$

12. The accurate area range for calculation of human Ac-globulin activity requires the use of from 0.06 to 0.10 ml of plasma. The calculated area of an unknown plasma must be such that the corresponding volume of plasma falls within this range. To preclude the necessity of determining the actual area in order to find the proper range, however, recourse may be had to a range finder graph (Fig. 3). The area is plotted against the clotting time at a constant incubation time. For example, if 0.08 ml of an unknown human plasma gives a clotting time of approximately 70 seconds after an incubation time of 12 minutes, the corresponding area would be approximately  $80 \text{ cm}^2$  which is in the correct range. If however, the clotting time were 100 seconds, the corresponding area would be  $60 \text{ cm}^2$ , and therefore outside of the range. Redilution of plasma would be necessary.

The thromboplastin used in the present quantitative study is the initial fraction of the crude saline extract prepared from fresh beef lung(5). The extract is diluted 1-10, placed in a waterbath at  $56^\circ\text{C}$  for 30 minutes,

<sup>†</sup> Oxalate correction factor.

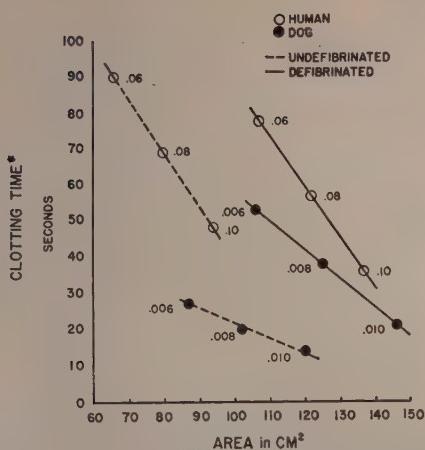


FIG. 3.

\*Clotting time at 12 min. incubation using undefibrinated plasma; at 6 min. incubation using defibrinated plasma.

and centrifuged for 10 minutes at 5000 r.p.m. The resulting solution is an easily prepared, clear, highly active thromboplastin without trace of prothrombin or Ac-globulin. It is stable at least 18 months when stored at -40°C. The use of this preparation was occasioned by the presence of small but significant amounts of Ac-globulin in some batches of purified thromboplastin prepared after the method of Chargaff *et al.*(6).

**Discussion.** The Ac-globulin activity in any given plasma is manifested as a function of 3 variables: (1) the time when prothrombin conversion begins, (2) the rate of prothrombin conversion, and (3) the yield of thrombin. Thus the area, circumscribed by the activation curve and by straight lines through a constant arbitrarily selected point on each axis (Fig. 1), is a measure of Ac-globulin activity which includes all three variables. A normal control plasma always should be assayed to determine the intercept on the reference curve. Although each or any combination of the three variables theoretically could be used as a criterion for the assay of Ac-globulin activity, our quantitative data

indicate that by using all 3 variables, a greater degree of accuracy is obtained.

The data used in the formulation of the reference curves for each species are based upon composite Ac-globulin assays of normal plasma from 33 humans, 15 dogs and 12 rats respectively. Four human and all rat assays were done on pooled plasmas of 4-5 each. The curves shown in Fig. 2 represent the linear component of sigmoid curves within a range of from 6 to  $10 \times 10^{-2}$  ml for human, and from 6 to  $10 \times 10^{-3}$  ml for dog and rat plasmas. Within this range, the slopes are relatively constant. Sixty-seven per cent of the human slopes varied less than 10%; 88% varied less than 15%; 87% of the dog slopes, and 92% of the rat slopes varied less than 10%. The average slope for human and dog non-defibrinated plasma and for human, dog, and rat defibrinated plasma was 1.5. Non-defibrinated rat plasma gave a slope of 0.75. With rat plasma, however, twice as much oxalate was used to prevent clotting as compared with other species. When the same amount of oxalate is used, the slope approaches 1.5, but clotting of plasma may occur. When 4 times as much oxalate is used, the slope approaches 0.37. Thus it would appear that the slope of the curves is at least partially dependent upon the amount of oxalate. For this reason, the amount of oxalate should be controlled carefully.

As the activity of Ac-globulin increases or decreases, the position of the sigmoid curves varies on coordinate paper. If the calculated areas remain within the range of 70 to 150  $\text{cm}^2$ , however, the slopes of the linear segments of the sigmoid curves remain constant enough so that these segments may be used as the basis for formulating the reference curves. As this range is exceeded, the slopes of the curves tend to deviate significantly. Of even greater importance than the area range, however, is the volume range. Irrespective of what the area range may be, it is essential that the volumes of plasma used vary from 6 to  $10 \times 10^{-2}$  for human, and from 6 to  $10 \times 10^{-3}$  for dogs and rats. From experience, we have found that below this range, the slopes vary appreciably, and that the curves deviate significantly from a

5. Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Exp. Med.*, 1937, v66, 801.

6. Chargaff, E., Moore, D. H., and Bendich, A., *J. Biol. Chem.*, 1942, v145, 593.

linear function. We attribute this to an obscure factor(s) which in some manner probably interferes with the activity of Ac-globulin. Our data suggest that this factor may be fibrinogen concentration. In a previous publication(7) and in quantitative data to be published, the importance of fibrinogen on Ac-globulin activity has been stressed. In constructing the reference curves from the normal control plasmas, it was observed that the maximum deviation of areas as well as the variation of slopes became progressively less as the accurate volume range was approached. Moreover, the maximum deviation of areas and slopes was decidedly less with defibrinated than with undefibrinated plasma. When 1 to 5 x 10<sup>-2</sup> ml of human plasma were used, the slopes of the lines derived from undefibrinated plasma were consistently less than those from defibrinated plasma. As the volume of plasma was increased to the accurate range, the slopes of the undefibrinated and of the defibrinated plasma approximated each other. Thus, the observations would indicate that fibrinogen alters the Ac-globulin activity and therefore the slopes of the reference curves, and that Ac-globulin assays can be done more ac-

7. Carter, J. R., and Warner, E. D., *PROC. SOC. EXP. BIOL. AND MED.*, 1950, v74, 388.

curately on defibrinated plasma.

Variables, such as temperature, reactivity of the biologic reagents etc. which affect other assay methods in blood coagulation, likewise exert their influence on the assay of Ac-globulin activity. The influence of these variables is manifested as a shift in intercept of the reference control curves from day to day. These variables do not alter significantly the slope of the reference curves. The existence of such variables, however, does demand that the intercept be established each day by the assay of Ac-globulin activity on normal control plasma.

In the assay of Ac-globulin activity, the prothrombin concentration is adjusted so that 2 units rather than 1 unit are present in the clotting tube. As a result, a smaller volume of plasma is required for the estimation of Ac-globulin activity, and the effect of anti-thrombin on the conversion rate and thrombin yield is correspondingly decreased.

*Summary.* A method for the quantitative estimation of Ac-globulin in plasma has been described. The method is based upon the use of three variables—conversion rate, thrombin yield, and onset of thrombin formation. With the use of all three variables combined, a greater degree of accuracy can be obtained.

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## Effects of B Vitamins and Liver on Growth of Immature Rats Maintained at Low Temperatures.\* (18153)

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Available data indicate that requirements for a number of nutrients are markedly in-

creased under conditions of low environmental temperature. An increased requirement for thiamine(1,2), riboflavin(3), pyridoxine(4),

\* This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number 314 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or endorsement of the Department of the Army.

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2. Ershoff, B. H., *Arch. Biochem.*, 1950, in press.
3. Mitchell, H. H., Johnson, B. C., Hamilton, T. S., and Haines, W. T., *J. Nutrition*, 1950, v41, 317.
4. Gyorgy, P., *J. Nutrition*, 1938, v16, 69.
5. Dugal, L. P., and Therien, M., *Canadian J. Res.*, 1947, v25, E, 111.

ascorbic acid(5), and vitamin A(6) has been demonstrated in animals maintained for prolonged periods of time at a low environmental temperature. Available data indicate that requirements may be increased for other nutrients as well. Smith *et al.*(7) observed that desiccated whole liver counteracted in part the growth retardation of immature rats maintained at low environmental temperatures. The protective factor was distinct from any of the known B vitamins (with the possible exception of vitamin B<sub>12</sub> which had not been isolated at that time). In the present communication data are presented on the comparative effects of various liver fractions and the known B vitamins on the growth and testicular weight of immature rats maintained under conditions of low environmental temperature.

**Procedure and results.** Five experimental rations were employed in the present experiment: diets A,B,C,D and E. Diets A and B were purified rations containing the B complex factors in synthetic form only. Diets C,D and E were similar in composition but contained desiccated whole liver, water-insoluble liver residue and water-soluble liver concentrate 1-20 in addition to the synthetic vitamins. One hundred and twenty male rats of the Long-Evans strain were selected at 25 to 28 days of age and an average weight of 59.5 g for the present experiment. Animals were kept in metal cages with raised screen bottoms to prevent access to feces and were fed *ad libitum* the diets listed in Table I. Experiments were conducted (1) with animals kept continuously in a large walk-in refrigerator at a temperature of  $2 \pm 1.5^{\circ}\text{C}$  and (2) under standard laboratory conditions at an average temperature of approximately  $23 \pm 2^{\circ}\text{C}$ . The dietary groups consisted of 16 rats each in the cold room series and 8 animals per group in the room temperature series. Animals were autopsied after 45 days of feeding, and testes, ventricular and adrenal weights determined.

Findings are summarized in Table II. Gain

6. Ershoff, B. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v74, 586.

7. Smith, E. D., Ershoff, B. H., Winzler, R. J., and Deuel, H. J., Jr., *J. Nutrition*, 1948, v35, 39.

TABLE I. Composition of Experimental Diet.

Dietary component	Diet A	Diet B	Diet C	Diet D	Diet E
Sucrose	60	60	50	50	57.5
Casein*	25	25	25	25	25
Cottonseed oil (Wesson)	10	10	10	10	10
Salt mixture†	5	5	5	5	5
Whole dried liver powder‡			10		
Extracted liver residue§				10	
Liver conc. powder 1-20					2.5

To each kg of diets A, B, C, D, and E were added the following synthetic vitamins: Thiamine hydrochloride, 40 mg; riboflavin, 40 mg; pyridoxine hydrochloride, 40 mg; niacinic acid, 60 mg; calcium pantothenate, 80 mg; 2-methyl-naphthoquinone, 10 mg; and choline chloride, 2 g. In addition to the above each kg of diet B was supplemented with the following synthetic vitamins: Thiamine hydrochloride, 20 mg; riboflavin, 20 mg; pyridoxine hydrochloride, 20 mg; niacinic acid, 60 mg; calcium pantothenate, 60 mg; p-aminobenzoic acid, 400 mg; inositol, 800 mg; folic acid, 10 mg; biotin, 4 mg; and vit. B<sub>12</sub>, 30 meg. Each rat also received once weekly 4.5 mg of alpha-tocopherol acetate and a vit. A-D concentrate containing 150 U.S.P. units of vit. A and 15 U.S.P. units of vit. D.¶

\* Vitamin test casein, General Biochemicals, Chagrin Falls, O.

† Hubbel, Mendel and Wakeman salt mixture, General Biochemicals, Chagrin Falls, O.

‡ Whole dried liver powder, Armour and Co., Chicago, Ill.

§ Extracted liver residue, Wilson Laboratories, Chicago, Ill. This material consists of the coagulated, water-insoluble material remaining after the removal of the extractable water-soluble substances.

|| Liver concentrate powder 1-20, Wilson Laboratories, Chicago, Ill. This material consists of the water-extractable material of raw liver.

¶ Nopeco fish oil concentrate, assaying 800,000 U.S.P. units of vit. A and 80,000 U.S.P. units of vit. D per g.

in body weight was significantly reduced in all rats under cold room conditions. Animals fed the added B vitamins (diet B) and those receiving the liver fractions (diets C, D and E) gained significantly more weight, however, than those fed diet A. Similar findings were obtained in respect to gonadal weight. The testes of rats fed diet A under cold room conditions were significantly smaller than those of animals fed diets B, C and D and to a lesser extent diet E. Under room temperature conditions, however, the gain in body weight of rats fed diet A did not differ significantly from that of animals fed diets B

TABLE II. Summary of Experimental Data.  
16 rats per group in cold room series; 8 animals  
per group in room temperature series.

Dietary group	Initial body wt, g	Gain in body wt over 45 day period,* g	Testes wt, * g
Cold room series			
A	61.4	90.1 ± 4.8 (14)	1.08 ± .14
B	60.7	120.6 ± 6.5 (14)	2.10 ± .15
C	60.1	115.8 ± 4.6 (16)	2.10 ± .12
D	60.4	118.8 ± 4.7 (15)	1.98 ± .12
E	60.9	116.9 ± 5.5 (15)	1.68 ± .07
Room temp. series			
A	56.9	177.7 ± 12.3 (8)	2.30 ± .16
B	57.1	186.1 ± 11.9 (8)	2.77 ± .10
C	55.9	236.5 ± 9.6 (8)	3.02 ± .09
D	57.6	221.7 ± 12.6 (8)	3.01 ± .11
E	56.7	184.7 ± 7.6 (8)	2.52 ± .20

The values in parentheses indicate the number of animals which survived and on which averages are based.

\* Including standard error of the mean calcu-

lated as follows:  $\sqrt{\frac{\sum d^2}{n}} / \sqrt{n}$  where "d" is the deviation from the mean and "n" is the number of observations.

and E, although the growth increment was increased in rats fed whole liver (diet C) or extracted liver residue (diet D). The gonads were well developed in all rats in the room temperature series. The weight of the testes in rats fed diet A was smaller than that of animals fed diets B, C, D and E; differences between the various groups, however, were not as marked as in the cold room series. Ventricular and adrenal weights when expressed in terms of mg per 100 g body weight were significantly increased in all rats in the low temperature series, averaging 440.2 mg and 18.3 mg respectively for animals under cold room conditions in contrast to 312.0 mg and 11.4 mg for rats maintained at room temperature. Differences between the various dietary groups, however, were not statistically significant.

*Discussion.* Available data indicate that

in addition to the major nutrients, there are substances present in our diet which may be required in increased amounts during conditions of stress. Such factors are apparently dispensable under normal conditions or their requirements are so small they may readily be met by amounts present in the diet or through the synthetic activity of the intestinal flora or the animals' own tissues. Body requirements for such nutrients may be increased by such factors as physical exertion, increased caloric intake, fever, thyroid-feeding and other conditions resulting in an increased metabolic rate. Such "stress factors" raise body requirements beyond the usual or average range, accentuate deficiencies and hasten the onset of symptoms on diets that would otherwise be adequate in the absence of the particular stress factor employed (8). Present findings indicate that prolonged exposure to cold is such a stress factor.

Under room temperature conditions diet A is an adequate diet for growth and gonadal development in the rat. Unpublished work from this laboratory indicates that male rats raised to maturity on this ration sire apparently normal young and female rats exhibit regular estrus cycles and give birth to living young although the lactation performance is poor. Present findings indicate that under room temperature conditions supplements of the known B vitamins (diet B) did not augment the gain in body weight of immature male rats over that obtained on diet A although gonadal weight was somewhat larger on the supplemented ration. In the cold room series, however, both body and gonadal weight were significantly greater on diet B. These findings indicate that prolonged exposure to cold increased requirements for one or more nutrients in diet B which were either absent or present to a smaller extent in diet A. Since these diets differed only in their B vitamin content, it would appear that the protective effect of diet B was due to its B vitamin content. Whole liver (diet C), liver residue (diet D), and liver concentrate 1-20 (diet E) also promoted growth and gonadal develop-

ment of immature male rats under cold room conditions. The protective effect of these supplements, however, was no greater than that provided by the synthetic B vitamin supplements. Since previous findings(7) indicate that supplements of the known B vitamins (excepting vit.  $B_{12}$ ) were without significant effect on the growth of immature rats fed a ration similar to diet A it would appear that the vit.  $B_{12}$  content of diet B was responsible at least in part for its protective effect under conditions of low environmental temperature.<sup>†</sup>

**Summary.** A marked retardation in body and gonadal weight was observed in immature male rats fed a purified ration under conditions of low environmental temperature. Supplements of desiccated whole liver, water-insoluble liver residue or water-soluble liver

extract resulted in a marked increase in both body and gonadal weight. A supplement of the known B vitamins was similarly effective. It is suggested that the protective effects of the various supplements were due, at least in part, to their vitamin  $B_{12}$  content.

<sup>†</sup> According to data supplied by the manufacturers Whole Dried Liver Powder contained approximately 1.1 mcg vit.  $B_{12}$ -like activity per g, Extracted Liver Residue from 0.2 to 0.4 mcg/g and Liver Concentrate Powder 1-20 from 3.0 to 5.0 mcg/g. Since the whole liver and liver residue were fed at a 10% level in the diet and the liver concentrate at a 2.5% level, the vit.  $B_{12}$  content of diets C,D and E were approximately 110 mcg per kg of diet, 20 to 40 mcg per kg of diet and 75 to 125 mcg per kg of diet respectively.

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### Toxicity and Effect of $\omega$ -Methylpantothenic Acid on Blood-Induced Infections of Plasmodium Lophurae in the Chick.\* (18154)

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The inhibitory activity of certain analogs of pantothenic acid toward a variety of organisms has attracted attention during recent years. Several analogs, notably derivatives of pantoyltauramide and phenylpantothenone, have been found active for some strains of plasmodia(1,2). The relatively high bacteriostatic effect of  $\omega$ -methylpantothenic acid

[N-(*a*,  $\gamma$ -dihydroxy- $\beta,\beta$ -dimethylvaleryl)- $\beta$ -alanine](3) prompted the present investigation on the antiplasmodial potentialities of this compound. The toxicity of this compound for the test animal, the chick, was extended to include its effect on rats, mice and on *Tetrahymena geleii* H.

**Toxicity studies. Chicks.** Preliminary experiments on a total of 20 chicks, 1-2 weeks old and weighing 53-85 g, showed no toxicity of single doses of sodium  $\omega$ -methylpantothenate given orally in concentrations as high as 8.4 g/kg body weight, intravenously in concentrations up to 0.4 g/kg and intraperitoneally in concentrations up to 1.0 g/kg. The analog (80% pure) was administered as a neutral 30% solution. Ten birds injected intraperitoneally with higher single doses (3.2-4.5 g/kg) exhibited torpor, loss of appe-

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<sup>‡</sup> Present address: Biology Department, California Institute of Technology, Pasadena, Calif.

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TABLE I. Response of *Tetrahymena gelei* H to  $\omega$ -Methylpantothenic Acid.

Sodium $\omega$ -methyl- pantothenate, $\gamma$ /tube	Calcium pantothenate, $\gamma$ /tube	Incubation time in days							
		Slanted tubes			Upright tubes				
		6 O.D.*	9 O.D.	14 O.D.	9 O.D.	14 O.D.	17 O.D.	21 O.D.	
0	0	.80	1.11	1.39	.45	.93	1.14	1.20	
20	0	.77	1.09	1.33	.54	.99	1.13	1.18	
200	0	.87	1.24	1.70	.42	.90	1.12	1.12	
2000	0	.48	.97	1.55	.40	.72	1.05	1.13	
20000	0	.28	.42	0.76	.21	.30	0.44	0.67	
0	5				.42	0.90	1.15	1.16	
20	5				.66	1.04	1.20	1.17	
200	5				.58	0.97	1.18	1.16	
2000	5				.54	0.97	1.23	1.26	
20000	5				.30	0.53	0.80	1.01	

\* Optical density  $\times 10$ , measured with a Lumetron photoelectric colorimeter, model 400A, using a red ( $650 \text{ m}\mu$ ) filter and corrected for the inoculated tubes at zero time. Values given are averages of duplicate tube readings.

† Added aseptically (0.1 ml) on the 6th day.

tite and inability to control equilibrium but all of the chicks recovered after 8-10 hours. Daily intraperitoneal injections of 0.45-0.50 g/kg for 5 successive days produced no demonstrable toxicity but when a larger dose (3.2-4.4 g/kg) was given to 5 birds for 6 successive days, 3 of the chicks died within 7 days and the remaining 2 succumbed on the 11th and 15th days. The average increase in weight of the treated birds on the 6th day was 16% compared to 35% for the controls. Reticulocyte counts of the treated chicks were not increased appreciably over the normal percentage of reticulocytes found in the peripheral blood of untreated 1-2-week-old chicks.

**Rats.** Eight rats of the Long-Evans strain 5-6 weeks old and weighing 120-150 g were given a single dose of sodium  $\omega$ -methylpantothenate orally via a catheter at a level of 10 g/kg body weight. The pure analog (N, calcd. 5.49; found, 5.46) was dissolved in an equal weight of water and the solution (pH, 10.1) was administered without being neutralized. Depilation was observed at sites where the solution accidentally came in contact with the fur. The toxic manifestations observed were rapidly-developing torpor, loss in appetite for many hours and diarrhea which was mild in 3 cases and severe in 5 others. Some of the experimental animals failed to gain weight in 3-5 days and others lost weight. A single intraperitoneal injec-

tion of the pure analog was administered unneutralized as a 10% solution (pH, 10.3) at a level of 1.0 g/kg to 8 rats 7-9 weeks old and weighing 180-240 g. This resulted in reduced activity but no other symptoms. On the other hand, following supplementation of a pantothenic acid-deficient diet with the analog at a level of 0.5%, marked loss in weight and death of weanling 28-day rats occurred within 2-3 weeks. This effect was prevented by inclusion in this diet of calcium pantothenate (.01%).

**Mice.** Eight young albino mice of the Bagg strain weighing 20-22 g recovered completely after a single oral dose of sodium  $\omega$ -methylpantothenate at a level of 10 g/kg. The analog was prepared and administered in the same manner as for rats. The torpor was somewhat less marked than that observed with rats. There were no marked toxic symptoms when the analog was given once intraperitoneally to 8 Bagg mice at a level of 0.9 g/kg or to 10 animals at 1.0 g/kg. A deficiency syndrome resulting from the daily ingestion of the analog was prevented or reversed by pantothenic acid(4).

**Protozoa.** The inhibition of growth of *Tetrahymena gelei* H by  $\omega$ -methylpantothenic acid was reversed by pantothenic acid as shown in Table I. The procedure and basal

TABLE II. Effect of Intraperitoneal Administration of  $\omega$ -Methylpantothenic Acid on *P. lophurae* in Chicks.

No.	Parasitemia in days (parasitized cells/10,000 rbc)							
	2	3	4	5	6	7	8	9
Experimental								
A1	570	1210	2280	3520	2560	2250	130	—50
2	620	1400	2480	3820	2730	1320	140	—50
3	740	1620	3060	4540	3320	1610	210	—50
4	480	1050	2020	2980	2350	920	110	—50
5	540	1180	2260	3340	2430	1220	140	—50
Avg	590	1292	2420	3640	2675	1244	146	—50
Control								
CA1	650	1520	2700	4100	2920	1410	160	—50
2	580	1310	2420	3580	2710	1230	130	—50
3	720	1550	2980	4420	3380	1520	190	—50
4	630	1450	2620	3780	2900	1300	150	—50
5	450	1170	1900	2800	2100	1150	90	—50
6	710	1590	2940	4360	3120	1450	200	—50
Avg	638	1431	2593	3640	2855	1343	153	—50

medium (supplemented with the analog as indicated) was that of Rockland and Dunn (5).<sup>§</sup>

*Effect of  $\omega$ -methylpantothenic acid on *P. lophurae* in chicks. Intraperitoneal administration.* The second day after inoculation with *P. lophurae* a 2 g/kg dose of the analog was administered twice daily for 4 successive days to 5 week-old chicks maintained on commercial starting chick mash. The strain of *Plasmodium lophurae* used was furnished by Dr. R. H. Rigdon then of the University of Arkansas School of Medicine. It was received in ducklings and maintained by transfer at 6-day intervals to week-old chicks. 0.2 ml of heparinized blood from chicks with at least 40% of red cells parasitized was used for inoculation. Growth and activity were decreased but there was no significant depression of parasitemia compared to 6 control chicks infected simultaneously with a similar inoculum (Table II). Infections, on the other hand, were markedly depressed by oral (400 mg/kg/day) or intraperitoneal (80 mg/kg/day) administration of 0.1% quinine hydrochloride.

5. Rockland, L. B., and Dunn, M. S., *J. Biol. Chem.*, 1949, v179, 511.

<sup>§</sup> The medium was modified by omitting pantothenic acid and liver extract and by increasing the level of amino acids about 50%. The authors are indebted to Dr. L. B. Rockland and Mr. Jose Lieberman for suggestions and assistance.

*Oral administration.* The analog (10 g/kg) was administered orally to 5-week-old chicks on the day preceding inoculation with *P. lophurae* and treatment with the analog (5 g/kg/day) was continued for 5 successive days. No depression of parasitemia was observed in the treated compared to the (5) control chicks.

*Effect of pantothenic acid deficiency on *P. lophurae* in chicks.* The inactivity of  $\omega$ -methylpantothenic acid toward *P. lophurae* suggested further study regarding the metabolic need of the parasite for pantothenic acid. Trager(6) demonstrated that pantothenic acid deficiencies do not alter the course of infections with *P. lophurae* in chicks and ducks, but these birds also had a concomitant biotin deficiency. Brackett, Waletzky and Baker(2) on the other hand, reported that trophozoite-induced infections of *P. gallinaceum* in chicks were strikingly less severe in pantothenate-deficient birds than in supplemented control chicks, suggesting that the blood phases of gallinaceum malaria require pantothenic acid for growth and multiplication. Inasmuch as the analog in question has been found to be active only against organisms which require pantothenic acid preformed in their media(7), it was thought expedient to repeat a portion of Trager's work by subjecting

6. Trager, W., *J. Exp. Med.*, 1943, v77, 557.

7. Drell, W., and Dunn, M. S., *J. Am. Chem. Soc.*, 1946, v68, 1868.

TABLE III. Blood-induced Infections of *P. lophurae* in Pantothenic Acid-Deficient Chicks.

Diet*	Age in days	Wt in g	% parasitized red cells in indicated days							
			2	3	4	5	6	7	8	9
A	15	63(7)†	3.5 (7)	6.0 (7)	12.4 (7)	28.0 (7)	21.0 (7)	12.0 (7)	2.0 (7)	-1.0 (7)
B	15	63(5)	3.0 (5)	6.8 (4)	12.0 (3)	31.0 (3)	24.0 (3)	10.0 (2)	1.0 (1)	-1.0 (1)
C	15	102(5)	5.0 (5)	7.9 (5)	16.5 (5)	36.1 (5)	38.0 (5)	25.0 (5)	10.0 (5)	5.0 (5)

\* Symbols defined in text.

† Figures in parentheses indicate number of birds on which averages are based.

pantothenic acid-deficient chicks to infection with *P. lophurae*. The 3 diets employed as indicated in Table III were (a) Diet A, essentially the vitamin-free diet of Brackett *et al.*(2), (b) Diet B, Diet A containing 1% of  $\omega$ -methylpantothenic acid and (c) Diet C, Diet A containing 0.6 mg% of calcium D-pantothenate. Although the concentration of pantothenic acid was slightly below the optimal according to Hegsted and Riggs(8), significant deficiency effects probably would not occur during the short time of the experiment. Each group of one-day-old chicks was fed one of the three diets *ad libitum*. Eight of the 12 birds on the deficient diets exhibited by the 15th day eyelid symptoms symptomatic of pantothenic acid deficiency. The weight of the chicks on the experimental diets averaged only about half of that of the animals on the control (C) diet. On the 15th day; the control and deficient chicks were inoculated intravenously with the parasite in doses commensurate with the weights of the birds. The basic dosage was 0.2 ml of blood with 40% R.B.C. infected, for a chick of 50-60 g. Four of the 5 chicks on the analog-supplemented deficient diet died within 22 days after initiation of the regimen.

As shown in Table III, the number of parasitized red cells was only slightly decreased in the birds on the deficient Diet A during the early stages of the parasitemia. By the sixth day, however, the chicks on both diets A and B had a lower percentage of parasitized cells than did those on diet C.

*Discussion.* It is noteworthy that sodium  $\omega$ -methylpantothenate has extremely low acute

8. Hegsted, D. M., and Riggs, T. R., *J. Nutr.*, 1949, v37, 361.

TABLE IV. Acute Toxicity\* of Calcium Pantothene and Sodium  $\omega$ -Methylpantothenate.

Animal	Na $\omega$ -methyl-pantothenate		Cal. pantothenate†	
	Oral	Intra-peritoneal	Oral	Intra-peritoneal
Mouse	10	.92	>10	>1
Rat	>10	.82	>10	>1
Chick			>8.4	>4.5

\* LD<sub>50</sub> in g/kg body wt.

† Data of Unna and Greslin(9).

toxicity‡ (Table IV). The rapid recovery of the animals after single large doses of the analog indicates a high rate of excretion. Levels high enough to interfere with the utilization of the tissue pantothenic acid can be maintained only by continued ingestion of the compound. Simultaneous supplementation with pantothenic acid prevents all symptoms associated with the analog, and addition of the vitamin during the course of the drug administration reverses its effects. It appears, therefore, that any inherent toxicity of the analog is of minor significance and that the observed effects are explained by interference with the utilization of the vitamin.

The response of *Tetrahymena geleii* H to  $\omega$ -methylpantothenic acid and its reversal by the vitamin probably signifies a requirement for pantothenic acid since to date few exceptions have been noted in *in vitro* experiments to the finding that reversible susceptibility to the analog is correlated with a vitamin re-

‡  $\omega$ -methylpantothenic acid at high levels is non-toxic to lactic acid bacteria in media containing adequate pantothenic acid(3).

9. Unna, K., Greslin, J. G., *J. Pharm. Exp. Therap.*, 1941, v73, 85.

quirement(10). The lack of response of the organism to added pantothenic acid and the relatively high level of analog required to cause inhibition indicate that the pantothenic acid or an active derivative in the natural supplement (cerophyl extract) of the medium was adequate for growth. These experiments were carried out prior to and agree with the report of Kidder and Dewey(11) on strain W of *T. geleii*.

Numerous possible anti-plasmodial agents have been tested following the report of Trager(12) that the *in vitro* survival of *Plasmodium lophurae* was prolonged by calcium pantothenate. Phenylpantothenone has been found to be the most effective analog of the vitamin in protecting chicks from such infections(1). Although it inhibited the growth of all microbial species tested, its effect generally was reversed by pantothenic acid only in organisms requiring the preformed vitamin (13).

According to Novelli and Lipmann(14, 15) both phenylpantothenone and  $\omega$ -methylpantothenic acid interfere with the synthesis of coenzyme A. Both compounds possess similar inhibitory activities toward lactic acid bacteria(3,13). On the basis of these results, therefore, the protective action of phenylpantothenone against *P. lophurae* in the chick does not appear to be due to interference with pantothenic acid utilization itself. It is still possible that this compound may interfere with the formation of coenzyme A but at a level not subject to reversal by the vitamin and not susceptible to interference by  $\omega$ -methylpantothenic acid. It has been observed in studies by Brackett *et al.*(2) that the percentage increase of immature erythrocytes produced in the chick by analogs of pantoyltauramide was approximately propor-

tional to the activity of these compounds as anti-plasmodial agents for *P. gallinaceum*. These substances were inactive toward *P. lophurae* in the duck(2). It is of interest that  $\omega$ -methylpantothenic acid, which is also inactive against *P. lophurae*, failed to produce an increased reticulocyte count in the chick. Cooperative studies by the National Institute of Health have shown that the analog is inactive against *P. cathemerium* in canaries (500-600 mg/kg fed twice daily for 4 days) and against *P. gallinaceum* in week-old chicks (in maximum tolerated doses(1) of 150 mg/kg fed twice daily for 4 days).

The decrease in parasitemia in deficient birds indicated in Table III is possibly due to the lack of pantothenic acid in these diets but this cannot be considered conclusive because of the few animals surviving and the difficulty in making accurate counts in the severely deficient and anemic birds. Becker *et al.*(16) showed that *P. lophurae* was able to mobilize pantothenic acid and/or biotin at the expense of the host. Hence even though blood levels of the vitamin decrease to 25% of normal in deficient chicks(17), this concentration may still be sufficient for initial growth, in contrast to *P. gallinaceum*, whose growth is markedly inhibited in deficient chicks. Trager(6) using *P. lophurae* in chicks found no significant difference between control and pantothenic acid-deficient birds. The heavier infection in birds with calcium pantothenate added to the diet was attributed to a partial biotin deficiency.

**Summary.**  $\omega$ -Methylpantothenic acid has been shown to exhibit low acute toxicity toward rats, mice and chicks. Growth inhibition of *Tetrahymena geleii* H at high levels of this analog was reversed by pantothenic acid. *Plasmodium lophurae* infections in chicks on the other hand were unaffected by  $\omega$ -methylpantothenic acid administered intraperitoneally at a level of 2 g/kg twice daily for 4 days or orally at 5 g/kg daily for 5 days. In chicks on a pantothenic acid-defi-

10. Drell, W., Dissertation, Univ. of Calif., Los Angeles, 1949.

11. Kidder, G. W., and Dewey, V. C., *Arch. Biochem.*, 1949, v21, 66.

12. Trager, W., *J. Exp. Med.*, 1943, v77, 411.

13. Woolley, D. W., and Collyer, M. L., *J. Biol. Chem.*, 1945, v159, 263.

14. Novelli, G. D., and Lipmann, F., *Fed. Proc.*, 1948, v7, 177.

15. Novelli, G. D., personal communication.

16. Becker, E. R., Brodine, C. E., and Marousek, A. A., *J. Infect. Dis.*, 1949, v85, 230.

17. Snell, E. E., Pennington, D., and Williams, R. J., *J. Biol. Chem.*, 1940, v133, 559.

cient diet. the simultaneous administration of  $\omega$ -methylpantothenic acid did not alter the

course of the infection with *P. lophurae*.

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### Effect of Feeding Aureomycin to Fattening Lambs. (18155)

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The recent work of Stokstad *et al.*(1) with chicks provided the first indication that a growth promoting factor in addition to vit. B<sub>12</sub> was produced by *Streptomyces aureofaciens*. More recently Stokstad *et al.*(2), in further work with chicks, found that aureomycin produced a growth response when added to diets containing vit. B<sub>12</sub> and B<sub>12b</sub>. Similar findings have been reported by Cunha *et al.* (3) and Jukes *et al.*(4) with swine. On the other hand, Colby *et al.*(5), in work with young lambs, found that an animal protein factor concentrate containing aureomycin was harmful. In the trial reported herein, it was decided to test the effects of adding vitamins to the rations of lambs fed aureomycin\* in an attempt to overcome these harmful effects. The effect on vitamin levels was indicated in the earlier work with lambs since the blood levels of vitamin B<sub>12</sub> were found to be lower in those lambs fed the APF concentrate containing aureomycin.

1. Stokstad, E. L. R., Jukes, T. H., Pierce, J., Page, A. C., Jr., and Franklin, A. L., *J. Biol. Chem.*, 1949, v180, 647.

2. Stokstad, E. L. R., and Jukes, T. H., *PROC. SOC. EXP. BIOL. AND MED.*, 1950, v73, 523.

3. Cunha, T. J., Burnside, J. E., Buschman, D. M., Glasscock, R. S., Pearson, A. M., and Shealy, A. L., *Arch. Biochem.*, 1949, v23, 324.

4. Jukes, T. H., Stokstad, E. L. R., Taylor, R. R., Cunha, T. J., Edwards, H. M., and Meadow, G. B., *Arch. Biochem.*, 1950, v26, 324.

5. Colby, R. W., Rau, F. A., and Couch, J. R., *Am. J. Physiol.*, in press.

\* The crude aureomycin and vitamins B<sub>12</sub> and B<sub>12b</sub> used in this study were obtained through the courtesy of Dr. T. H. Jukes and Lederle Laboratories, Pearl River, N. Y.

**Experimental.** Eighteen lambs of mixed breeding weighing an average of about 58 lb were used in this study. They were divided into 3 lots of 6 on the basis of weight and sex. The lambs in Lot I received the basal ration consisting of 90% milo, 10% cotton-seed meal plus alfalfa hay, free choice. The lambs in Lot II received the basal ration plus sufficient crude aureomycin to furnish 100 mg of pure aureomycin, daily by capsule. The lambs in Lot III were fed the basal ration plus 100 mg of aureomycin daily by capsule and in addition received an allowance of B-vitamins fed orally by capsule every second day. The B-vitamins fed were as follows (per lamb daily, based on the quantities reported by Lindley *et al.*) (6); thiamine 6.8 mg, riboflavin 1.6 mg, niacin 15.6 mg, pantothenic acid 6.5 mg, pyridoxine 2.6 mg, choline 130 mg, biotin 34  $\mu$ g, folic acid 169  $\mu$ g, paraaminobenzoic acid 16.9 mg, and inositol 167 mg. In addition a mixture of vit. B<sub>12</sub> and B<sub>12b</sub> was fed at the rate of 20  $\mu$ g per lamb daily. Feed and weight records were kept. At the close of the trial, 2 lambs from each of the 3 lots were slaughtered, their stomach contents sampled and total bacteria counts made. Bacterial counts were made by the tryptose agar plate method. The cultures were incubated at 37½°C and the total count made at the 48th hour period of incubation.

**Results and discussion.** The gains and feed efficiency of the 3 lots of lambs used in this trial are shown in Table I. It may be noted that the lambs in Lot I made very satisfactory gains (0.52 lb per head per day).

6. Lindley, C. E., Brugman, H. H., Cunha, T. J., and Warwick, E. J., *J. An. Science*, 1949, v8, 590.

TABLE I. Effect of Aureomycin on Weight, Gains, and Feed Consumption of Lambs.

Lot No.	I	II	III
Supplement fed	.	Aureomycin	Aureomycin + B-vitamins
Avg initial wt of lambs	54.66	58.30	58.75
Avg daily gain or loss	0.52	- .16	-.20
Avg daily feed consumption, lb.			
Concentrates	1.50	.10	.10
Alfalfa hay	1.25	.20	.18

TABLE II. Effect of Aureomycin Supplementation on Rumen Bacteria Counts and Character of Rumen Contents of Lambs.

Sheep No.	Character of rumen content	Bacterial count per g rumen content, $\times 100,000$
4903 control	Large rumen, contents coarse, moist, fermented with gas formation	5
5293 "	Same	7.5
4904 aureomycin	Intermediate size rumen, contents coarse, moist, fermented with gas formation	22
4908 "	Intermediate size rumen, contents coarse, dry, and small amount of gas formation	8
4906 "	Small rumen, contents fine and quite liquid, no gas formation	64,000
4901 aureomycin + B-vit.	Same	61,000

during the 14 days of the trial. The lambs that received aureomycin alone lost an average of 0.16 lb per head per day while the lambs fed the aureomycin plus B-vitamins (Lot III) lost an average of 0.2 lb per head daily. The trial was of short duration because the two groups of lambs fed the aureomycin were losing weight so rapidly, it was decided to conclude the tests rather than sacrifice the lambs. The lambs in Lot I consumed 1.5 lb of the concentrate mixture daily plus 1.25 lb of alfalfa hay. In contrast, the lambs in Lot II ate only an average of 0.10 lb of concentrates and 0.20 lb of alfalfa hay each day. The lambs in Lot III ate 0.10 lb of concentrates and 0.18 lb of alfalfa hay daily. It may be noted that no beneficial effect was derived from the vitamin supplement fed because those lambs receiving this supplement plus aureomycin ate less feed and lost more weight than the lambs fed aureomycin alone.

The bacterial counts and rumen character of the lambs, as shown in Table II, indicates quite a difference between the 3 lots of lambs. The control animals had a large rumen well filled with feed of normal appearance. In

addition these animals had a low bacterial count (500,000 and 750,000 bacteria per gram) for perhaps two reasons: (1) the rumen of these control animals was well filled with feed and hence made a certain "dilution" factor for the bacterial counts and (2) there was probably a normal competitive environment between the various kinds of bacteria which tended to keep counts low. In the groups of animals (Lot II) receiving aureomycin alone, the rumen was somewhat smaller and contained much less feed. The contents were, however, of fairly normal character. Bacterial counts were much higher; 8,000,000 and 22,000,000 bacteria per gram of rumen contents. This may indicate that the aureomycin had destroyed certain strains of bacteria, thus eliminating the competitive environment and permitting other less desirable strains of bacteria to grow more rapidly. The animals in Lot III had the highest bacterial counts (61,000,000,000 and 64,000,000,000 per g). It is most likely that the rumen requires certain essential strains of bacteria for it to digest food and function properly. These essential bacteria may have been destroyed by the aureomycin and the

vitamins may have aided the growth of other less desirable strains. This is further indicated since the feed in the rumen of the animals in Lot III was finely divided and quite liquid, indicating that it had been in the rumen for some period of time, since the rumen was not functioning properly.

The findings in this trial lend further support to the earlier work of Colby *et al.*(5). The previous work might be criticized because the lambs had included in their ration an APF supplement containing aureomycin. This might have caused the ration to be unpalatable and hence reduced feed consumption and gains. In this trial, the aureomycin was fed orally by capsule and all lambs received the identical ration. The aureomycin must have produced such harmful effects within the lambs, probably by its effect on the rumen flora, to cause the animal to lose its appetite and thus result in weight losses. It is further

postulated that the effect of the aureomycin was not due to the loss of vitamins normally supplied by bacterial synthesis, since feeding vitamins produced no beneficial effects. The findings during this trial and the previous trial reported by Colby *et al.*(5) indicate that aureomycin has a deleterious effect on lambs.

**Summary.** The feeding of 100 mg of aureomycin daily to lambs resulted in markedly decreased feed consumption and weight loss. Bacterial counts of rumen contents of lambs fed aureomycin and aureomycin plus vitamins was much higher than control animals, indicating that perhaps the aureomycin had destroyed certain strains of bacteria, thereby, eliminating a normal competitive environment and permitting less desirable strain of bacteria to multiply.

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### The Vitamin B<sub>12</sub> Requirement of the Syrian Hamster.\*† (18156)

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The white rat has been proposed as an assay animal for estimating the vit. B<sub>12</sub> potency of foods and liver extracts(1-3). For these tests iodinated casein and/or sulfaguanidine were added to a soybean oil meal or casein basal ration, and a depletion period used to accentuate a vit. B<sub>12</sub> deficiency. Avail-

able information, although contradictory in certain instances(4,5), indicates that the dietary requirement of the hamster for certain factors may be greater than for the white rat. Since studies on the requirement of the hamster for vit. B<sub>12</sub> have not been reported, it was of interest to determine the vit. B<sub>12</sub> requirement with the use of different basal rations. Further, if the hamster was shown to have a definite vit. B<sub>12</sub> requirement, an additional animal species would be available for assaying the vit. B<sub>12</sub> potency of various materials.

**Experimental and results.** Four basal rations were used in the preliminary investigations: a corn-soybean ration with and without

\* We are indebted to Wilson Laboratories for supplying the Whole Liver Substance, to Merck and Co. for the vitamin B<sub>12</sub> and National Oil Products Co. for the vitamin A and D concentrate used in these studies.

† Journal Paper No. 31, American Meat Institute Foundation.

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2. Lewis, U. J., Register, U. D., Thompson, H. T., and Elvehjem, C. A., *Proc. Soc. Exp. BIOL. AND MED.*, 1949, v72, 479.

3. Frost, D. V., Fricke, H. H., and Spruth, H. C., *Proc. Soc. Exp. BIOL. AND MED.*, 1949, v72, 102.

4. Schweigert, B. S., *Vit. and Hormones*, 1948, v6, 55.

5. Granados, H., and Dam, H., *Fed. Proc.*, 1950, v9, 360.

iodinated casein, and a casein ration with and without iodinated casein.<sup>†</sup> The composition of the corn-soybean oil meal rations was as follows (in percent): ground yellow corn 40.64, soybean oil meal 50, corn oil 4.4, cystine 0.3, fortified cod liver oil 0.6 (400 D and 2250 A per g), Salts IV(6) 4, and iodinated casein 0.06, when added. For the casein ration, 24% casein, 63.67% sucrose, and 3% cellulflour replaced the ground yellow corn and soybean oil meal. The B vitamins (except vit. B<sub>12</sub>) and 2-methyl-1, 4-naphthoquinone were added to all rations in amounts described in previous studies with hamsters (7). Food and water were provided *ad libitum*. The corn-soybean oil meal ration is similar to that described by Register, *et al.* (1); however, the amount of soybean oil meal has been increased and all the vitamins were added as supplements in the present studies. Male Syrian hamsters were purchased<sup>‡</sup> for the initial experiments. The initial weight of these animals averaged 51.5 g. They were distributed into four groups and fed the basal rations indicated for a 2-week depletion period. A high mortality was observed during this period for those animals which received the casein ration plus iodinated casein; consequently, the corn-soybean oil meal ration plus iodinated casein was fed to this group for the remainder of the experiment. After the 2-week depletion period, each group was divided into 3 equal sub-groups. The animals for these sub-groups were selected as uniformly as possible on the basis of weight and gain in weight for the previous 2-week test. Each sub-group received either the basal ration, the basal ration plus 50 µg of vit. B<sub>12</sub> per kg of ration, or 2% whole liver substance (added at the expense of the entire ration). This level of vit. B<sub>12</sub> was selected since in our experience with white rats, 50 µg per kg would provide 3-5

TABLE I. Studies on the Vitamin B<sub>12</sub> Requirement of the Hamster.

Basal ration	Supplement	No. of animals	Avg wts	
			Initial	Final†
Corn-soybean + iodinated casein	0	12	64	97
	50 µg B <sub>12</sub> /kg	12	62	95
	2% W.L.S.‡	12	63	97
Corn-soybean + iodinated casein*	0	7	67	95
	50 µg B <sub>12</sub> /kg	7	66	101
	2% W.L.S.	7	67	101
Corn-soybean	0	12	67	94
	50 µg B <sub>12</sub> /kg	12	65	88
	2% W.L.S.	12	66	93
Casein	0	9	66	101
	50 µg B <sub>12</sub> /kg	9	66	90
	2% W.L.S.	9	67	101

\* The depletion ration for this group was the casein + iodinated casein ration (see text).

† After 4 wk on experiment.

‡ Whole liver substance.

times the requirement with the use of similar basal rations. Two per cent whole liver substance was chosen as a supplement which may provide additional unknown factors, if any, required by the hamster as well as vit. B<sub>12</sub> (estimated 40-60 µg per kg of ration), and at a level which would increase the total protein content of the ration by only a small amount. The number of animals in each group, the initial weights after depletion, and the final weights after 4 weeks on experiment are shown in Table I.

The results of this experiment show that a vit. B<sub>12</sub> requirement of the hamster could not be demonstrated. Although a slight improvement in the performance was noted when vit. B<sub>12</sub> was added for one of the groups, either no difference in growth or slightly less growth was observed for the other 3 groups receiving vit. B<sub>12</sub> as compared to the basal groups. Also, no increase in gain was noted with the addition of 2% whole liver substance, which showed that at this level of intake no additional unknown factors were provided when either the corn-soybean oil meal or casein basal ration was used. An inspection of the data for the animals that were smaller at the start of the experiment showed that the growth rates were similar to those for the groups as a whole, which indicated that the initial

‡ Protamone, purchased from the Cerophyl Laboratories.

6. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, v138, 459.

7. Schweigert, B. S., McBride, B. H., and Carlson, A. J., *PROC. SOC. EXP. BIOL. AND MED.*, 1950, v73, 427.

§ Haskins Rabbitry, St. Louis, Mo.

weights of the animals, within the limits available for the study, were not a factor in affecting the response to the ingestion of vit. B<sub>12</sub>.

Since in our own experience, as well as that of others(1,2), rats develop a definite vit. B<sub>12</sub> deficiency with the use of a similar dietary regimen, a further study was conducted to compare the growth of white rats and of hamsters when fed the corn-soybean oil meal plus iodinated casein basal diet with and without crystalline vit. B<sub>12</sub>. Weanling, male, white rats<sup>1</sup> and weanling hamsters (28 g) were used for this study. The latter were obtained from our stock colony which was maintained on rations designed to avoid an excessive intake of vit. B<sub>12</sub>. The same ingredients were used in the preparation of the diets since it was possible that in the first experiment the amount of vit. B<sub>12</sub> in the basal rations may have been sufficiently high so that a vit. B<sub>12</sub> requirement could not be demonstrated.

All animals were fed the basal ration for a depletion period of 2 weeks. On the basis of the performance during this period uniform groups of rats were selected and fed the following supplements: 1—none; 2—50 µg of vit. B<sub>12</sub> per kg; 3—2% whole liver substance; and 4—50 µg of vit. B<sub>12</sub> per kg with the food intake restricted to the average intake of Group 1. The food efficiency for Groups 1, 2, and 4 was also determined. The hamsters were fed the same diets; however, the group with the restricted food intake was omitted. The results for this experiment are shown in Table II.

These studies confirm the findings of the first experiment with hamsters in that no requirement for vit. B<sub>12</sub> or of additional unknown factors could be shown. A definite vit. B<sub>12</sub> deficiency was demonstrated for the rat, however, with the use of identical diets. No additional response was observed when the liver supplement was added. The food efficiency of the basal group was 0.20, for the vit. B<sub>12</sub> supplemented group (fed *ad libitum*) 0.26, and the supplemented group (food intake restricted) 0.21.

**Discussion.** Although a vit. B<sub>12</sub> requirement could not be demonstrated for the hamster with the use of experimental conditions

TABLE II. Growth of Hamsters and Rats Fed a Corn-Soybean-Iodinated Casein Ration Plus Various Supplements.\*

Supplement to basal diet	White rat		Hamster	
	No. per group	g grain/wk	No. per group	g grain/wk
None	7	21.2	7	8.0
50 µg B <sub>12</sub> /kg	7	38.7	4	8.6
2% W.L.S.	7	36.4	5	8.1
50 µg B <sub>12</sub> /kg†	6	22.2		

\* 4-week period for the rats and a 6-week period for the hamsters.

† Food intake restricted to that for the group receiving no supplement.

equivalent to that of the rat, it is recognized that with other conditions a requirement may be demonstrated. It would appear that a higher initial store of the vitamin was not a complicating factor in the present study since weanling animals from our colony, without a large intake of vit. B<sub>12</sub>, also failed to show a vit. B<sub>12</sub> requirement. It is apparent, therefore, that if the hamster does require dietary source of vit. B<sub>12</sub>, its requirement is much less than that of the white rat. From these results and those reported recently(5) it appears likely that the hamster does not have greater dietary requirement for vitamins than the white rat as indicated by a summary of earlier studies(4). On the basis of these findings, the hamster is not a suitable test animal for the determination of the vit. B<sub>12</sub> potency of foods.

The high mortality observed in the first experiment for hamsters fed the casein ration plus iodinated casein is in accord with results obtained with rats(8,9). These findings support the conclusions of Ershoff(10) and others(8,11) that soybean oil meal and liver contain an anti-thyrotoxic factor(s) not pres-

8. Lewis, U. J., Tappan, D. V., Register, V. D., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 568.

9. Scheid, H. E., and Schweigert, B. S., (unpublished data).

10. Ershoff, B. H., *J. Nutrition*, 1949, v39, 259.

11. Betheil, J. J., and Lardy, H. A., *J. Nutrition*, 1949, v37, 495.

ent in sufficient quantities in the purified casein rations.

**Summary.** A vit. B<sub>12</sub> requirement was not demonstrated for the Syrian hamster when fed either a corn-soybean oil meal ration with or without iodinated casein, or a casein basal ration with or without iodinated casein. No increase in the rate of gain was observed when

2% whole liver substance was added to these rations. Parallel experiments were conducted with rats fed the corn-soybean oil meal ration plus iodinated casein in which a vit. B<sub>12</sub> requirement was demonstrated. A reduction in food efficiency was observed in rats attributable to the vit. B<sub>12</sub> deficiency.

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## Anticonvulsant Properties of Benadryl and Pyribenzamine.\* (18157)

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It is well known that antihistaminic drugs, such as diphenhydramine (Benadryl) and tripelennamine (Pyribenzamine), produce side effects by acting on the central nervous system. According to Feinberg(1) the most consistent side effect of all antihistaminic drugs is sedation. The clinical implication of this effect has been recognized, particularly in relation to the use of antihistaminic agents in the treatment of diseases referable to the central nervous system.

The salutary effect of Benadryl in paralysis agitans was reported by McGavack and co-workers(2). Subsequently, Budnitz(3) called attention to the beneficial effect of this drug in Parkinson's disease. These observations have since been confirmed(4-7) and extended to include other antihistaminic agents(4,6,7).

McGavack and coworkers(2) also tried Benadryl in 3 patients with grand mal epilepsy, and reported an increased frequency of seizures as a result of this medication. More recently, Churchill and Gammon(8) have shown that Benadryl diminished the electrical abnormality and decreased the incidence of seizures in petit mal; on the other hand, both Pyribenzamine and Benadryl induced seizures in some patients with grand mal epilepsy. Such reports on the use of antihistaminic drugs are particularly interesting when one considers that convulsions may occur when large doses of these agents are ingested(9-11).

In view of the wide use of antihistaminic agents and because of the limited information on their central nervous system actions, it was thought important to study their anticonvulsant effects in experimental animals. It was anticipated that such a study might not only provide information on the anticonvulsant properties of these drugs but might also indicate the validity of employing them in the treatment of allergic manifestations in epileptic patients.

**Methods.** Male albino rats obtained from

\* This investigation was supported by a research grant from the National Institutes of Health, Public Health Service.

1. Feinberg, S. M., *Ann. New York Acad. Sc.*, 1950, v50, 1186.

2. McGavack, T. H., Elias, H., and Boyd, L. J., *Am. J. M. Sc.*, 1947, v213, 418.

3. Budnitz, J., *New England J. Med.*, 1948, v238, 874.

4. Berger, F. M., *New York State J. Med.*, 1949, v49, 1817.

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6. Gair, D. S., and Ducey, J., *Arch. Int. Med.*, 1950, v85, 284.

7. Effron, A. S., and Denker, P. G., *J. Am. M. A.*, 1950, v144, 5.

8. Churchill, J. A., and Gammon, G. D., *J. Am. M. A.*, 1949, v141, 18.

9. Duerfeldt, T. H., *Northwest. Med.*, 1948, v46, 781.

10. Lehman, G., *J. Pharm. and Exp. Therap.*, 1948, v92, 249.

11. Rives, H. F., Ward, B. B., and Hicks, M. L., *J. Am. M. A.*, 1949, v140, 1022.

TABLE I. Anticonvulsant Potency of Benadryl and Pyribenzamine in Comparison with Dilantin.\*

Drug	Route	Toxicity (TD <sub>50</sub> ) mg/kg	Max. electroshock test†	
			Effective dose (ED <sub>50</sub> ) mg/kg	Protective index (P.I.)
Benadryl	Oral	193 (146-255)	125 (112-140)	1.5 (1.1-2.1)
	i.p.	18.5 (13.7-24.9)	11.0 (10.2-11.8)	1.7 (1.2-2.3)
Pyribenzamine	Oral	450 (378-535)	143 (124-164)	3.1 (2.5-3.9)
	i.p.	29.0 (26.4-31.9)	13.0 (9.7-17.4)	2.2 (1.7-3.0)
Dilantin	Oral	>3200	30.0 (20.0-45.0)	>100
	i.p.	82 (55-122)	11.3 (8.8-14.5)	7.3 (4.5-11.8)

\* All values in parentheses indicate 95% confidence limits.

† 150 m A., 0.2 second, alternating current, corneal electrodes.

Dilantin and Benadryl were kindly supplied by Dr. Oliver Kamm, Parke, Davis and Co.; Pyribenzamine, by Dr. Jock L. Graeme, Ciba Pharmaceutical Products.

the Sprague-Dawley farm were used as experimental animals. The substances examined in this study were given both orally and intraperitoneally. When the oral route of administration was employed, the drug was suspended in 10% acacia mucilage and given by stomach tube. In either case the assay procedures were carried out at the previously determined time of peak drug effect. The details of the assay procedures have been previously published(12). Briefly, anticonvulsant potency (ED<sub>50</sub>) of each drug was determined by its ability to abolish the tonic extensor component of maximal seizures (maximal electroshock seizure test) and to protect the animals from the subcutaneous administration of 70 mg/kg of pentylenetetrazol (Metrazol test). In addition, minimal neurological toxicity (such as loss of placing responses, ataxia, depression, etc.,) was determined (TD<sub>50</sub>), and protective indices (P.I.=TD<sub>50</sub>/ED<sub>50</sub>) were calculated. To permit critical comparison of potencies, the data obtained were evaluated by the method of Litchfield and Wilcoxon(13).

**Results.** The results obtained are shown in Table I.

It may be seen that Benadryl and Pyri-

bazine are more toxic than diphenylhydantoin (Dilantin). Benadryl is the most toxic of the 3 compounds studied. When given by the intraperitoneal route, 18.5 mg/kg produced minimal toxic symptoms in 50% of rats as compared to 29.0 mg/kg for Pyribenzamine and 82 mg/kg for Dilantin. It was impossible to estimate toxicity after the oral administration of Dilantin because of poor absorption of this drug from the gastro-intestinal tract. We have previously called attention to the fact that only a small percentage of the maximum dose administered (3200 mg/kg) is actually absorbed when the oral route of administration is employed(14).

No remarkable difference was observed in the antielectroshock potency of Benadryl and Pyribenzamine as compared to Dilantin. Thus, when administered intraperitoneally, 11.0 mg/kg of Benadryl abolished the tonic extensor component in 50% of rats as compared to 13.0 mg/kg for Pyribenzamine and 11.3 mg/kg for Dilantin. By the oral route Dilantin was found to be 4 to 5 times as potent as Benadryl or Pyribenzamine.

No protection was afforded by these compounds against Metrazol convulsions. Indeed, all animals receiving Pyribenzamine or large doses of Benadryl appeared exquisitely sensitive to Metrazol and had severe, recur-

12. Swinyard, E. A., *J. Am. Pharm. A.* (Sc. Ed.), 1949, v38, 201.

13. Litchfield, J. T., Jr., and Wilcoxon, F., *J. Pharm. and Exp. Therap.*, 1949, v95, 99.

14. Swinyard, E. A., and Toman, J. E. P., *J. Pharm. and Exp. Therap.*, in press.

rent and often fatal seizures.

*Discussion.* Everett(15) reported that Benadryl and Pyribenzamine did not effect the "electroshock threshold for full tonic clonic seizures." The observations were made on mice given intraperitoneal doses of 10 to 50 mg/kg. Our data were obtained in rats and indicate that these same drugs have a potency almost as great as Dilantin when tested for their ability to modify maximal electroshock seizure pattern. The reason for the difference in the results reported here and those of Everett is unknown, unless it be a species difference.

It is interesting to compare the observed differences between Benadryl and Pyribenzamine. Benadryl is approximately twice as toxic as Pyribenzamine but the toxicity patterns differ for the two drugs. Benadryl has a wide range between the doses causing hyperexcitability and spontaneous seizures. Some differences were also observed when these drugs were tested against Metrazol. Neither Benadryl nor Pyribenzamine had the ability to prevent Metrazol convulsions, but small doses (5 to 10 mg/kg) of Benadryl did modify seizure pattern. In contrast, Benadryl in large doses and Pyribenzamine in all doses employed appeared to synergize with Metrazol to cause severe, protracted and often lethal convulsions.

The data indicate that Benadryl, Pyribenzamine and Dilantin have several properties in common. All 3 drugs modify maximal electroshock seizure pattern in non-toxic doses but do not prevent Metrazol convulsions. Toxic doses of all three drugs produce increased excitability and convulsions. We have previously called attention to the fact that clinically useful antiepileptic hydantoins and barbiturates are characterized in the laboratory by their ability to modify maximal electroshock seizure pattern(16). Inasmuch as Benadryl and Pyribenzamine have a maximal electroshock seizure potency approaching that of Dilantin, it would be of interest to know whether they are effective clinically in grand mal. The only data we have been able to

find concerning the effects of antihistaminics in grand mal are those of McGavack and co-workers(2) and Churchill and Gammon(8). McGavack and coworkers tried Benadryl in 3 cases of grand mal and reported a "definite aggravation of status" in each instance. Churchill and Gammon employed Benadryl in 4 cases of grand mal and Pyribenzamine in one case of grand mal but concluded that the patients had "major seizures so infrequently" that the effect of the medication could not be evaluated. It would be most interesting to have additional clinical data on a larger series of patients with grand mal in order more precisely to determine the effect of these drugs. If such data substantiated the limited results reported to date it would then appear that Benadryl and Pyribenzamine have no value in grand mal and may actually aggravate such cases. This would suggest that the maximal electroshock seizure test may have questionable predictive value for antiepileptic efficacy when employed to measure anticonvulsant action of antihistaminic derivatives of ethanalamine and ethylenediamine.

It is tempting to speculate on the mechanism whereby Benadryl depresses the spike-wave abnormality, as reported by Churchill and Gammon(8). It has long been known that certain central stimulants, such as ephedrine, amphetamine and caffeine are effective in some cases of petit mal. Indeed, before the discovery of Trimethadione, Paradione and Phenurone, these agents were practically the only drugs useful in petit mal. The mechanism of antiepileptic action of central stimulants is unknown, but it is probably entirely unlike that of the central depressants. It is well established that sensory stimulation may occasionally abort cortical seizures(17,18) and that seizure discharges are more common in sleep than during the waking state(19). In

17. Jackson, J. H., Selected writings of John Hughlings Jackson. Vol. I, Epilepsy and Epileptiform Convulsions. Edited by James Taylor, Hodder & Stoughton, London, 1931.

18. Penfield, W. and Erickson, T. C. Epilepsy and cerebral localization. Charles C. Thomas, Springfield, Ill., 1941.

19. Gibbs, E. L., and Gibbs, F. A., Proc. Assn. Res. Nerv. and Ment. Dis., 1947, v26, 366.

15. Everett, G. M., Fed. Proc., 1950, v9, 270.

16. Swinyard, E. A., Fed. Proc., 1947, v6, 376.

view of these facts we have previously postulated that "it is not unlikely that a drug-induced increase in activity of normal brain may inhibit discharges from seizure foci"(20). The greater ability of Benadryl, as compared with Pyribenzamine, to stimulate the central nervous system in subconvulsive doses could account for the salutary effect of this drug in

20. Goodman, L. S., Toman, J. E. P., and Swinneyard, E. A., *Arch. internat. de pharmacodyn. et de thérap.*, 1949, v78, 144.

petit mal.

**Summary.** Benadryl, Pyribenzamine and Dilantin were tested in rats for toxicity and for ability to modify maximal electroshock seizure pattern and to prevent Metrazol convulsions. The data indicate that all three drugs modify maximal electroshock seizure pattern but do not prevent Metrazol convulsions. The significance of the data is discussed.

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## Influence of Methadone on Oxygen Uptake and Glycogenolysis of Rat Liver Slices.\* (18158)

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The effects of morphine-like analgetics, including methadone, on the metabolism of surviving tissues and isolated enzyme systems have been studied by several workers(1-4), in an attempt to explain the mechanism of action of analgetics. Although these studies have not accomplished their primary purpose, they show that analgetics affect tissue metabolism in a manner different from anesthetics, and they indicate the usefulness of methadone as a tool in the study of oxidative processes.

The present study was undertaken as a result of the observation that the same concentration of methadone increased the oxygen uptake of liver slices from fed rats but decreased that of slices from rats starved overnight. Since the effect was the same whether or not glucose was included in the suspending medium, the possibility of increased glycogenolysis by liver slices in the presence of methadone has been

investigated by biochemical and histological techniques.

**Methods.** Liver slices were obtained from adult albino rats of the Slonaker-Wistar strain. When high initial liver glycogen values were desired, the animals were starved for twenty-four hours and then given a mixture of half glucose, half ground stock diet overnight before use. Animals which had been starved overnight provided liver slices low in glycogen content. The rats were sacrificed by a blow on the head, the livers quickly removed, placed in a cold box(5) and with a Martin slicer(6) cut into slices approximately 0.5 mm thick. The slices were randomized and used either for measurement of oxygen uptake, chemical determination of glycogen or histologic examination. Oxygen consumption was measured by the direct method of Warburg(7) at 37.2°C. The liver slices which weighed 50-100 mg were suspended in 2 ml of a modified Krebs-Ringer phosphate buffer(4) with or

\* Supported in part by a grant from the National Institutes of Health, Bethesda, Md.

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3. Watts, D. T., *J. Pharm. and Exp. Therap.*, 1949, v95, 117.
4. Elliott, H. W., Warrens, A. E., and James, H. P., *J. Pharm. and Exp. Therap.*, 1947, v91, 98.

5. Fuhrman, F. A., and Field, J., 2nd, *J. Biol. Chem.*, 1944, v153, 515.

6. Martin, A. W., *Endocrinology*, 1942, v30, 624.

7. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric Techniques and Tissue Metabolism*, 2nd Edition, Burgess Publishing Co., Minneapolis, Minn., 1949.

without 0.2% glucose: (Extracellular Ringers = ER; with 0.2% glucose added = EGR.) Vessels containing tissue from either fed or starved animals were read at 15-minute intervals for 90 minutes to establish control QO<sub>2</sub>'s for each vessel. Then to half the vessels 0.005M methadone was added from the side arms of the Warburg flasks to make a final concentration of 0.0005M, and readings were continued for an experimental period of 90 minutes. The quantitative determination of glycogen was done by the method of van Wagtendonk *et al.*(8). Analyses were performed at death, at the time the Warburg vessels were placed in the constant temperature bath, and at the completion of each 180-minute run.

Liver slices from 2 fed animals were used for histologic examination of glycogen. No starved animals were included because the low glycogen content of their livers would make histologic differentiation of glycogen difficult. For the same reason, the 90-minute control period was omitted in the determination of oxygen uptake; methadone was added to half the vessels at zero time and QO<sub>2</sub>'s were measured on all vessels for a 90-minute experimental period. Control slices were fixed at the time the Warburg vessels were placed on the 37.2°C bath; all others after they had respired for 90 minutes in EGR with or without methadone. The fixative was ice cold Rossman's picro-alcohol-formalin mixture(9). After fixation 8 μ celloidin sections were stained with hematoxylin and Best's carmine and mounted.

**Results. Oxygen Uptake.** QO<sub>2</sub>'s were determined by the direct method of Warburg on liver slices from 10 fed and 6 starved animals. When the data were analyzed, it was apparent that the presence of glucose in the Ringer solution did not alter the QO<sub>2</sub>'s of either control or experimental slices; hence the results were combined and are presented graphically in Fig. 1. The oxygen uptake of slices from fed animals was increased immediately to a maximum of 127% of the control value at 120

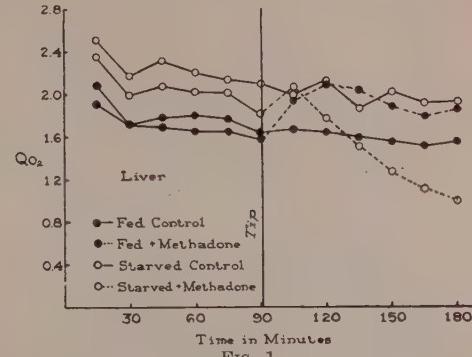


FIG. 1.

The effects of methadone on the oxygen uptake of liver slices from fed and starved rats. Each point represents the average of 29-39 slices from 10 animals in the case of the fed animals and 24 slices from 6 starved animals.

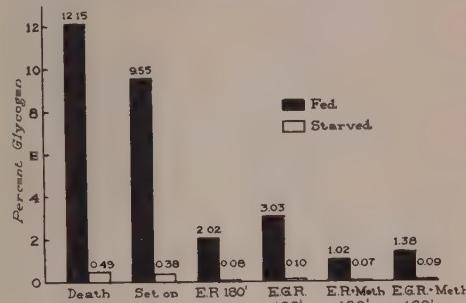


FIG. 2.

The glycogen content of liver slices from fed and starved animals. See text for explanation.

minutes, falling to 119% at 180 minutes; the latter value was found to be significant by comparing probable errors of the means ( $P < 0.001$ ). In contrast, methadone significantly inhibited the oxygen uptake of slices from starved animals; at 180 minutes the experimental value was 51% of the control ( $P < 0.001$ ). The higher control values for slices from starved as compared with fed rats may be attributed to the increased bulk of the fed livers resulting from the storage of glycogen and water(10), *i.e.*, metabolically inert weight.

**Glycogen Analyses.** The results of the glycogen analyses are graphically portrayed in Fig. 2. Overnight starvation depleted liver

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9. Rossman, I., *Am. J. Anat.*, 1940, v66, 277.

10. Fuhrmann, F. A., and Field, J., 2nd, *Arch. Biochem.*, 1945, v6, 337.

glycogen to relatively low values. Livers from 6 fed animals contained  $12.15 \pm 0.91\%$  (PE mean) glycogen falling to  $9.55 \pm 0.62\%$  in the 20-30 minutes required to prepare the slices for oxygen uptake studies. In the control vessels at 180 minutes glycogen content fell to  $2.02 \pm 0.12\%$  in ER and  $3.03 \pm 0.18\%$  in EGR. The glycogen sparing effect of glucose was significant ( $P = 0.001$ ). Methadone (0.0005M) significantly increased the rate of glycogenolysis in ER or EGR ( $P < 0.001$  in both cases). The glycogen content of methadone treated slices at 180 minutes was  $1.02 \pm 0.15\%$  in ER and  $1.38 \pm 0.16\%$  in EGR. The glycogen sparing effect of glucose was found to be not significant ( $P = 0.25$ ), but the trend was the same as in the control vessels.

*Histologic Examination.* Sections prepared from tissue which had respired in EGR for 90 minutes showed partial depletion of glycogen throughout some and peripheral exhaustion in all; glycogen was absent from the outer one or two cell layers. In the presence of methadone marked glycogen depletion was obvious throughout all sections and peripheral depletion extended deeper than in control slices. No damage to cellular structure could be detected.

*Discussion.* The ability of the same concentration of methadone to inhibit the oxygen uptake of liver slices poor in glycogen and increase the oxygen uptake of slices rich in glycogen suggests that the observed stimulation is actually a manifestation of enzyme inhibition. According to McElroy(11), it is generally accepted that, in various organisms,

if oxidative phosphorylation is inhibited, inorganic phosphate will increase to a concentration which will permit a rapid breakdown of stored material such as glycogen. The fact that under the conditions of our experiments the glycogen sparing effect of added glucose is apparent in the absence of methadone and is practically obliterated by 0.0005M methadone, coupled with the finding that methadone can inhibit hexokinase(1) suggests that such a mechanism may be operating here. However, such a succinct explanation must be viewed with caution since a large excess of inorganic phosphate is always available in the suspending medium. In addition, it has been shown that methadone increases the oxygen uptake of rat brain slices respiring in a medium containing glucose(4).

Preliminary experiments indicate that in bicarbonate buffer 0.0005M methadone does not stimulate glycogenolysis and inhibits oxygen uptake; hence, further studies are contemplated to investigate the role of ionic constituents of the suspending medium in producing the findings reported above.

*Summary.* 1. Methadone (0.0005M) increases the oxygen uptake of liver slices rich in glycogen and depresses it in slices poor in glycogen. 2. The increased oxygen uptake is accompanied by an increased rate of glycogenolysis as demonstrated by biochemical and histologic methods. 3. A possible explanation for the findings is discussed.

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## Comparative Reactivity of Isolated Surviving Coronary Arteries to L-Epinephrine and L-Norepinephrine.\* (18159)

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The comparative reactivity of isolated surviving coronary arteries to l-epinephrine and l-norepinephrine has not been determined. Recorded observations on the coronary flow response to norepinephrine include the perfused hearts of rabbits(1), cats (1,2) and dogs(3) as well as the dog heart-lung preparation(2) and the dog using the rotameter(4). All of these studies have demonstrated an increase in coronary blood flow associated with a concomitant increase in heart rate and amplitude of contractions(1-3). As pointed out by Folkow(3), in these studies it is difficult to determine whether true vasodilation has occurred, or whether the observed increase in coronary flow is due solely to the increased activity of the heart muscle. By these present studies of the isolated coronary arteries we have endeavored to determine whether l-norepinephrine and epinephrine have a direct vasodilating action on these vessels.

**Experimental Procedure.** The coronary arteries of healthy slaughtered swine were examined by the angioplethysmokymographic technique of Smith and Syverton(5,6). The vessels were removed and mounted in the plethysmograph and perfused with Tyrode's solution until the arterial wall had assumed a cer-

tain degree of tone. The artery was then tested with l-epinephrine (Winthrop-Stearns) and l-norepinephrine (kindly supplied by Winthrop-Stearns) by injecting test doses of usually  $50 \gamma$  of the drugs into the perfusate. This provided a stimulus of 5 parts per million for 15 seconds under these experimental conditions. Variation in the dosage down to  $1 \times 10^{-8}$  did not change the type of reaction observed. Volume changes of the artery were recorded as a measure of constriction or dilation.

**Results.** Eleven coronary arteries which had during perfusion assumed a certain degree of tone have been tested with l-norepinephrine. In each instance vasodilation resulted. Fig. 1 illustrates a typical experiment (No. 728). In 4 instances it was possible to compare the degree of vasodilation produced by l-epinephrine and l-norepinephrine in the same vessel by measurement of the areas of the reactions recorded by a planimeter.<sup>1</sup> These measurements expressed in square centimeters are recorded in Table I.

**Discussion.** l-epinephrine and l-norepinephrine cause vasodilation of the isolated surviving coronary arteries of swine. In those instances when it was possible to compare the effects of similar test doses of l-epinephrine

\* This study has been supported in part by a Research Grant from the Masonic Foundation for Health and Human Welfare.

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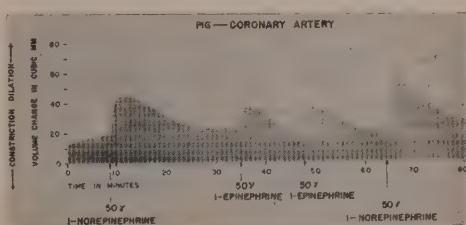


FIG. 1.

Exp. #728. Swine coronary artery. Comparative study of vasodilation produced by similar ( $50 \gamma$ ) test doses of L-epinephrine and L-norepinephrine.

TABLE I. Comparative Vasodilation of Coronary Arteries to L-epinephrine and L-norepinephrine.

Exp.	L-epinephrine	L-norepinephrine
714	1.7	3.5
728	4.0	10.0
728	4.3	10.5
729	2.2	5.8

and l-norepinephrine the nor compound produced vasodilation about  $2\frac{1}{2}$  times greater than l-epinephrine as measured by this technique. The molecular weight of norepinephrine is 92.3% that of epinephrine hence these studies were not accomplished on a strictly equimolecular basis, yet the differ-

ence in reactivity far exceeds the probable difference attributable to differing molecular weights.

*Summary.* 1. l-norepinephrine and l-epinephrine produce vasodilation of the isolated surviving coronary arteries of swine.

2. l-norepinephrine produces about  $2\frac{1}{2}$  times the degree of vasodilation produced by l-epinephrine in the same artery in the same dose. This difference exceeds the anticipated difference attributable to differing molecular weights.

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### Comparative Study of the Ketogenic Activity of Certain Niacin Compounds.\* (18160)

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Normal rats, when fed on a niacin deficient diet, probably synthesize sufficient niacin or niacin-like compounds to carry on normal metabolic processes, providing the diet contains adequate amounts of protein(1-3). However, when excessive amounts of niacin are given to diabetic rats or to normal fasting rats, a marked ketonuria is produced, resulting presumably from some alteration in the metabolism of fat(4-5). The present work is concerned with a comparison of the ketogenic action of niacin, niacinamide and the alcohol of

niacin (Roniacol) in the non-fasting diabetic rat.

*Experimental.* Twenty-one adult male rats of the Long-Evans strain, 8 to 10 months of age, were used. After the rats had been fasted for 48 hours, diabetes was produced by a single subcutaneous injection of 125 mg of alloxan/kg body weight. All of the rats were diabetic for at least 2 months before the experiment was started and most of the animals showed a severe diabetes. Qualitative urinary acetone body determinations were made daily by the sodium nitro-prusside method, which gave an estimation of the degree of ketonuria. In the conditions of this experiment, each rat acted essentially as its own control.

*Effect of niacin compounds when 10% fat diet was used.* Initially, the animals were given the basal diet†(5) for several days in order to determine a baseline for the excretion of urinary acetone bodies. After this was established they were fed an experimental diet which consisted of the basal diet with 0.1%

\* This investigation has been made with the assistance of a grant from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Assn.; also, by a grant from the Central Scientific Fund, College of Medicine, State University of Iowa.

1. Heidelberger, C., Abraham, E. P., and Lepkovsky, S., *J. Biol. Chem.*, 1948, v176, 1461.

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3. Rosen, F., and Perlzweig, W. A., *J. Biol. Chem.*, 1949, v177, 163.

4. Janes, R. G., and Myers, L., *Proc. Soc. Exp. BIOL. AND MED.*, 1946, v63, 410.

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† The basal synthetic diet contained 10% fat, 18% protein, 70% carbohydrate with essential vitamins and minerals.

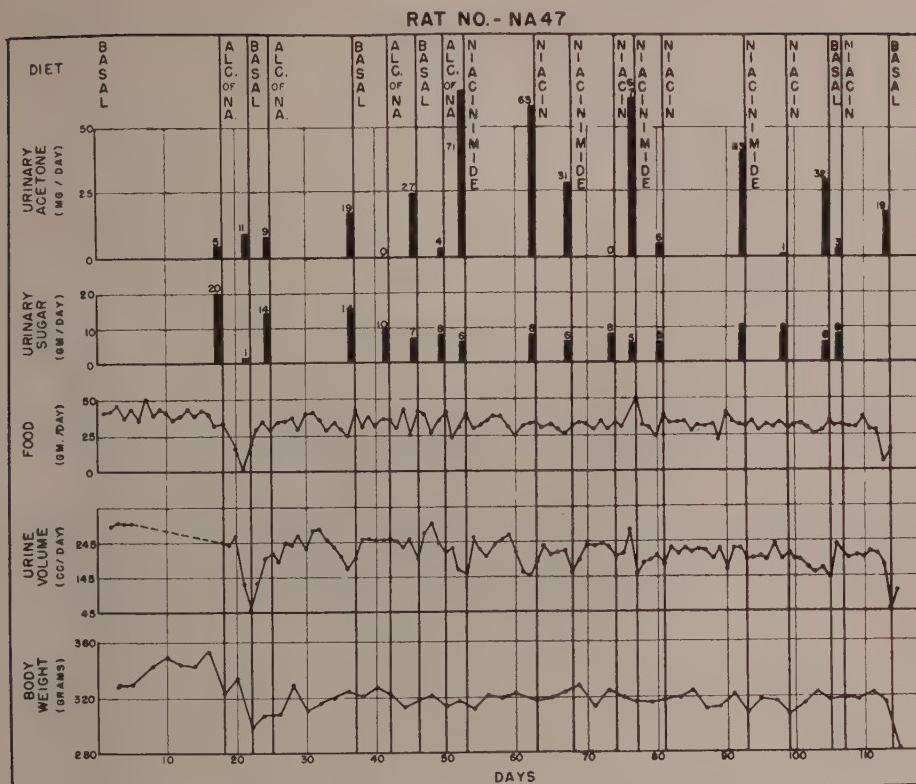


FIG. 1.

The alcohol of niacin, niacin and niacinamide diets were made by adding 0.1% of these compounds, respectively, to the basal diet. The values for urinary acetone and sugar are for the last 24 hours that a particular diet was fed.

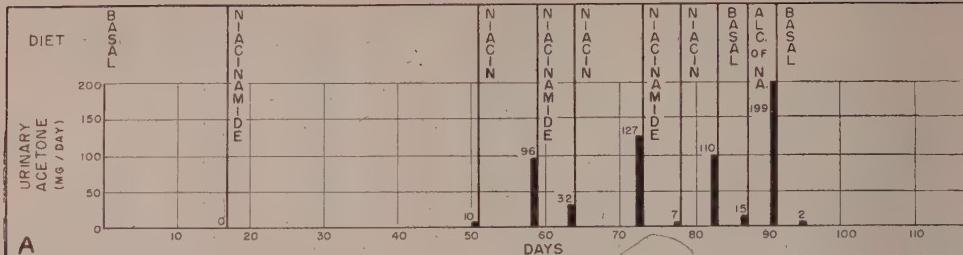
of niacin, the alcohol of niacin or niacinamide.<sup>†</sup> The experimental diets were alternated several times with the basal ration during the study. Fig. 1 and 2A illustrate the manner in which the diet was alternated in two of the rats. Usually some increase in the excretion of acetone bodies was noted while the rats were eating diets containing either 0.1% niacin or alcohol of niacin. In many instances, however, several periods of feeding with the niacin compounds were necessary before ketonuria of a pathological degree was seen. When the rats were returned to the plain basal diet for 1 or 2 days, the severe ketonuria disappeared. Usually several days on the basal

regimen were allowed to intervene between treatment with different compounds. However, since the niacinamide diet was the least effective in producing a ketonuria, in certain instances (Fig. 1), it was alternated with the diets containing 0.1% niacin or the alcohol of niacin rather than going back to the plain basal diet. Just before a diet was changed, quantitative urinary acetone body determinations were made by the method of Van Slyke and urinary sugar was determined by the micro-method of Somogyi(6). Of the 21 rats studied, 10 showed a rather prompt ketogenic response to niacin or the alcohol of niacin. Data for 2 animals, representative of the group, are shown on Figures 1 and 2A. When animal NA 47 received a supplement of 0.1%

<sup>†</sup> The vitamins were supplied through the kindness of Dr. Elmer L. Sevringshaus, Hoffmann-La Roche, Inc.

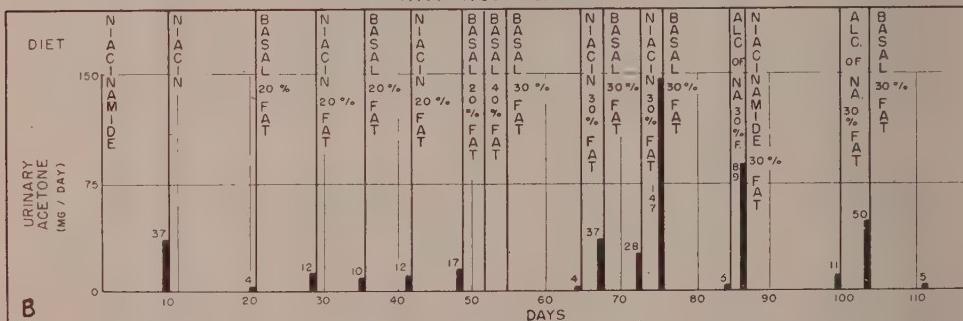
## KETOGENIC ACTIVITY OF NIACIN COMPOUNDS

RAT NO.-NA43



A

RAT NO.-NA44



B

FIG. 2A.

The diets fed to Rat No. NA43 were similar to those given No. NA47 (Fig. 1).

FIG. 2B.

The niacin compounds were added to the high fat diet in the proportion of 0.1 mg to 350 calories of diet.

alcohol of niacin a mild ketonuria became evident on the 37th day of the experiment, and a severe ketonuria was present on the 52nd day. This pathological state persisted for 10 days even though the rat received the ration containing 0.1% niacinamide. The sequence of the alternation of diets is shown in Fig. 1. Towards the end of the study, the rats appeared to have developed some refractiveness to the niacin compounds, since the latter were less effective in producing ketonuria.

It was impossible to detect any ketone bodies in the urine of the second rat (NA 43) during 17 days on the basal regimen. The addition of 0.1% niacinamide caused only small amounts of urinary acetone to be excreted after 33 days on the supplemented diet. However, as soon as 0.1% niacin or alcohol of niacin was introduced, a marked ketonuria was seen.

In addition to urinary acetone values, Fig. 1 shows values for urinary sugar, food intake, urine volume and body weight. These data

are quite typical for all the rats used in this experiment. The severity of the diabetes, as indicated by urine sugar values, declined during the course of the experiment. The curves for food intake, urine volume and body weight appeared very similar, since reduced urine volume and loss in body weight accompanied declines in food intake. No explanation can be given for the anorexia which occurred on the 21st day of the experiment. In certain instances, however, loss of appetite appeared to accompany marked ketonuria (not shown in Fig. 1). A reduced food intake is not the cause of the ketonuria, as was shown in previous studies in which rats fasted for as much as 5 days excreted only small amounts of acetone bodies(5).

*Effect of niacin compounds when 20 to 30% fat diet was used.* When niacin or the alcohol of niacin failed to evoke ketonuria within a period of 2 weeks, the rats under observation were given a diet containing a higher fat content(7), in order to make them mildly keto-

TABLE I. Excretion of Urinary Acetone Bodies. Average values last 24 hours when niacin compounds were given.

	No. diabetic rats	Controls	Niacinamide	Niacin	Alcohol of niacin
Basal synthetic diet	10	6.0(28)*	9.5(13)	27.4(26)	62.1(12)
20 to 30% fat diet	7	11.5(25)	21.7(3)	44.4(8)	72.0(13)

\* No. of determinations.

nuric or bring them to the borderline of ketosis. A diet containing 20% fat was first given and the fat content was increased up to 30% when necessary. The protein in the high fat diets was maintained at 19% of the total calories and extra vitamins and minerals were added in proportion to the caloric content in order to prevent deficiencies. One particular level of fat was fed until the excretion of ketone bodies became stabilized. Excessive amounts of the niacin compounds were then added to the diet. Eleven rats were placed on the high fat diets and 7 showed an increased excretion of acetone bodies when niacin or the alcohol of niacin was fed. Three rats responded while on the 20% diet, one on the 25% and 3 on the 30% fat diet. The rats which did not respond to these diets had a milder diabetes and thus could not be brought to the borderline of ketosis. The urinary acetone excretion of one diabetic rat, representative of the group, which was fed the high fat ration is shown on Fig. 2B. Originally this rat showed a mild ketonuria when given the basal diet plus niacinamide, but since niacin was not effective in elevating the urinary ketone bodies the high fat diet was introduced. The 20% fat diet, with or without 0.1% niacin, failed to induce a severe ketonuria but when 0.1% niacin was added to the 30% fat diet a marked ketonuria was produced. Likewise, the 0.1% alcohol of niacin was effective in increasing urinary acetone bodies when it was fed with the 30% fat diet.

There was considerable variation in the ketogenic response shown by the different rats in the niacin compounds. As has been indicated, in certain animals it was necessary to alternate the experimental and basal diets several times before a marked ketonuria de-

veloped. Also, in some cases, there seemed to be a peak in the response to the niacin compounds which was followed by a slow but usually gradual decline in the level of ketone body excretion. However, it was possible to compare the effectiveness of the 3 niacin compounds by taking averages of the acetone body excretion just before the diets were alternated. Table I gives average values for mg of acetone excreted in the 17 rats which showed the ketogenic response. Data are shown for those fed the basal and the high fat diets. It is quite apparent from these data that the alcohol of niacin is the most ketogenic, niacin the next, and niacinamide the least ketogenic.

Recently, Banerjee *et al.*(8) gave intravenous injections of 500 mg of niacinamide to normal and diabetic patients. There was no increase of blood ketone bodies during the following two hours. From the data presented in the present studies, however, one would not expect that niacinamide would cause much of a ketosis, particularly during a two-hour period. Unpublished studies from this laboratory have shown that a well-controlled human diabetic may tolerate 1800 mg of nicotinic acid daily, when given orally, without showing a significant rise in urinary acetone bodies.

**Summary.** Excessive amounts of niacinamide, niacin or the alcohol of niacin were given to 21 diabetic rats. The latter two compounds caused a marked increase in the excretion of ketone bodies in 10 of these animals. The 11 remaining rats were fed a diet containing 20 to 30% fat in order to make them mildly ketonuric or bring them to the borderline of ketosis. When excessive amounts of the niacin compounds were then added to the high fat diets, 7 of the rats

7. Janes, R. G., and Prosser, M., *Am. J. Physiol.*, 1947, v151, 581.

8. Banerjee, S., and Ghosh, N. C., *J. Biol. Chem.*, 1949, v177, 789.

showed an increase in acetone body excretion. It is apparent from the data presented that the alcohol of niacin is the most ketogenic,

niacin next and niacinamide the least ketogenic.

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## Effect of Variations in Osmotic Pressure on Macrophages in Tissue Culture.\* (18161)

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In experiments dealing with the cultivation of the exoerythrocytic forms of *Plasmodium gallinaceum* it became necessary to study the effects of variations of the culture medium. This in turn required a knowledge of the range of osmotic pressure tolerated by the malarial parasite and the host cell, the macrophage. This paper presents the results of experiments on the effect of variations in osmotic pressure on macrophages and parasites.

**Methods.** The tissue culture methods used in the present experiments have been described in detail(1). The macrophages were derived from chick embryo spleens infected with the exoerythrocytic stages of *Plasmodium gallinaceum*. Macrophages were grown *in vitro* at various constant levels of osmotic pressure for 4 days. These levels varied from 3 to 16 atmospheres of pressure at 37°C. The osmotic pressure determinations were made by means of the cryoscopic method. In previous experiments(1) it was determined that the optimal pH range for malarial parasites was pH 7.2 to 8.0, and for macrophages pH 6.8 to 8.2. For this reason the pH of the medium was kept between pH 7.4 and 7.8 in the present experiments. This was done by

means of a continuous flow of CO<sub>2</sub> gas mixtures. The nutrient medium consisted of chicken serum diluted with a balanced salt solution.

The osmotic pressure was varied in two ways: (1) by varying the water content only, keeping the relative proportion of ingredients constant, and (2) by varying the content of NaCl. In the first set of experiments hypotonic solutions were prepared by starting off with a mixture of chicken serum and Tyrode's solution and by the addition of varying amounts of water to produce the desired hypotonic solutions. To achieve similar results with hypertonic solutions, a starting hypertonic solution was made from a mixture of 50% chicken serum and 50% triple concentrated Earle's solution(2).<sup>‡</sup> Such a mixture has an osmotic pressure of 16 atmospheres at 37°C. Starting with this solution various hypertonic solutions varying from 16 to 8 atmospheres were made by the addition of water. In this set of experiments, however, the percentage of serum varied from one experimental group to another. This necessitated setting up a parallel series of control groups to determine the effect of diluting the serum at constant osmotic pressure. This was done by mixing various concentrations of serum in Tyrode's to match the experimental

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† Present address—Pathology Section, National Cancer Institute, Bethesda, Md.

1. Dubin, I. N., and Yen, C. K., *Arch. Path.*, in press.

2. Earle, W. R., *J. Nat. Cancer Inst.*, 1943, v4, 165.

‡ Triple concentrated Earle's solution was highly alkaline, giving a precipitate of salts. To avoid the precipitate the solution was sterilized by passing through a sintered glass filter under 10 lb pressure of 100% CO<sub>2</sub>. It was stored in stoppered bottles under equilibration with the same gas.

groups in which osmotic pressure was varied. This led to the observation that a high concentration of serum was inhibitory to the macrophages and the malarial parasites. This was confirmed in later experiments designed to test the effect of concentration of serum on the cells and parasites.

Consequently, a second set of experiments was set up in which the concentration of serum was kept constant at the optimal level, namely 20%. In this group of experiments osmotic pressure was varied by varying the concentration of NaCl. The chicken serum was mixed with Tyrode's solution which was split into 3 components, (a) a tenfold concentration of NaCl, namely 8.0% NaCl in H<sub>2</sub>O, (b) H<sub>2</sub>O, and (c) double strength Tyrode's solution from which NaCl was omitted.

**Results.** The results of the 2 groups of experiments were similar. This is not surprising since NaCl accounts for most of the osmotic pressure in serum and in a serum-Tyrode's mixture. Indeed there was not much difference in the final concentrations of sodium and chloride at any level of osmotic pressure achieved by either of the two methods.<sup>§</sup>

The optimal growth of macrophages occurred between 7 to 11 atmospheres of pressure. Fifty percent of growth occurred at 5 and 13 atmospheres. Growth ceased at 4 and 15 atmospheres. The parasites similarly exhibited optimal growth between 7 and 11 atmospheres. Fifty percent growth occurred at 6 and 12 atmospheres. There was no growth of parasites at 5 and 14 atmospheres.

Some morphological differences in the macrophages were noted as between the hypertonic and hypotonic solutions. When compared with normal cells, the macrophages in the hypertonic solutions were somewhat smaller and contained slightly less cytoplasm and fewer vacuoles; these cells tended to stain more sharply and brilliantly than the cells in the control group at 8.4 atmospheres. In the hypotonic solutions the cells presented a foamy appearance and the cytoplasm seemed

edematous. In addition the cells appeared to stick together, suggesting an increased cohesiveness. The nuclei of such cells were irregular and presented a ragged nuclear membrane.

**Discussion.** In the present experiments the optimal growth of macrophages at 37°C occurred between 7 to 11 atmospheres of pressure. White(3) found that in Locke-Lewis solution chick embryo tissues remained in good condition at osmotic values from 3 to 11 atmospheres when the ratio of ingredients was unchanged. Willmer(4) noted that chick embryo tissues (a mixture of epithelium and fibroblasts from intestines) showed optimal growth in concentrations of sodium chloride from 0.4% to 1% (representing a range of from about 4 to 10 atmospheres of pressure).

An interesting effect was that of the dilution of the serum in the culture medium. A high concentration of serum was found to be inhibitory to the macrophages and the malarial parasites. Ebeling(5) observed that the dilution of the tissue culture medium at constant osmotic pressure produced a more extensive zone of cell proliferation but no increase in actual mass of newly formed tissue. Lambert(6) also noted that dilution of plasma with isotonic solution caused a more extensive migration in cultures of cells of the actively migratory type, such as those of the spleen and bone marrow. Dilution with a limited quantity of distilled water produced the same effect. Less actively motile cells (e.g. epithelium) were influenced little or not at all by dilution. The effect on cells of the migratory type he considered to be due probably to the reduction in quantity of fibrin in the clot, producing lessened resistance to cell locomotion. This explanation appears improbable, however, in the light of the present experiments. In the experiments described in the present paper the macrophages were grown directly on the glass coverslips without the use of plasma clot, and here also the dilution of the culture medium resulted in

3. White, P. R., *Growth*, 1946, v10, 231.

4. Willmer, E. N., *Brit. J. Exp. Biol.*, 1927, v4, 280.

5. Ebeling, A. H., *J. Exp. Med.*, 1914, v20, 130.

6. Lambert, R. L., *J. Exp. Med.*, 1914, v19, 398.

<sup>§</sup> Values for sodium levels in chicken serum were kindly supplied by Dr. Richard Overman, Department of Physiology, University of Tennessee, Memphis.

an increased growth or migration. It seems more likely that the stimulating effect of the dilution of the serum resulted from a dilution of some inhibitory substance in the chicken serum.

**Summary.** 1. The optimal growth in tissue culture of macrophages and the exoerythrocytic forms of *Plasmodium gallinaceum* oc-

curred between 7 and 11 atmospheres of pressure. Growth was reduced to 50% at 5 and 13 atmospheres.

2. High concentrations of serum (over 50%) were inhibitory to both the malarial parasites and the host cell, the macrophage.

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## Changes in the Reactivity of Purified Prothrombin After Freeze Drying.\* (18162)

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In work on the purification of prothrombin it became evident that the protein could not be activated as readily as the prothrombin of native plasma. The question arose whether this might be due to an alteration in the reactivity of the prothrombin or to the removal of essential activators. When evidence for the latter possibility became available in abundance, there followed a general tendency to consider the question closed. However, the reactivity of prothrombin can be changed; for example, under proper conditions, thrombin can produce alterations(1). This altered prothrombin can still be converted to thrombin but not with calcium plus thromboplastin plus Ac-globulin.

We have now found that freeze drying of purified prothrombin preparations produces changes which cause the prothrombin to be refractory to the physiological activators. This makes it desirable to avoid the freeze drying technic when the object is to retain purified prothrombin for long periods of time. The change in reactivity of the purified prothrombin is not noticeable immediately after drying. The loss in activity occurs later, and

variable periods of time are required for extreme changes to take place. The sequence of events is approximately as follows: First the prothrombin becomes refractory to the activators such as calcium, thromboplastin, and Ac-globulin. Activation with sodium citrate, thrombin and 3-chloro-4,4'-diamino-diphenyl sulfone is then still possible. Later even this activation procedure is no longer effective and finally the dried prothrombin becomes insoluble in physiological saline solution.

**Experimental procedures.** Purified prothrombin was obtained from bovine plasma (2). The assay for prothrombin involved the use of the modified 2-stage procedure, which supplies Ac-globulin, calcium, and thromboplastin for the activation of prothrombin(3). Thrombin activity was measured by the method of Seegers and Smith(4). The apparatus used for drying prothrombin from the frozen state was similar to that described by Seegers(5).

**Time relationships.** The loss in activity of a large number of products has been studied

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\* This investigation was supported by a grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

1. Seegers, W. H., and McClaughry, R. I., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 247.

2. Seegers, W. H., McClaughry, R. I., and Fahey, J. L., Blood, 1950, v5, 421.

3. Ware, A. G., and Seegers, W. H., Am. J. Clin. Path., 1949, v19, 471.

4. Seegers, W. H., and Smith, H. P., Am. J. Physiol., 1942, v137, 348.

5. Seegers, W. H., Science, 1945, v101, 284.

TABLE I. Changes in Activity of Dried Purified Prothrombin.

Prothrombin product No.	Initial activity			
	Total units, X 1000	Specific*	% activity lost	Days after drying
470822	165	925	17	30
480218	105	1260	67	196
480322	231	1305	69	162
480519	167	1240	75	105
480527	180	1350	78	97
480810	215	1335	52	25
490125	300	1270	98	421
491215	465	1370	46	124
470422A	185	720	99	1065
480831	110	1065	98	568

\* Specific activity is here expressed in units per mg dry wt.

and representative data are assembled in Table I. All of the preparations had high specific activity, which was completely retained during the drying operation. After drying they were placed in a desiccator containing  $P_2O_5$ . The prothrombin activity was periodically measured quantitatively by the modified 2-stage procedure. The amount of activity lost varied from product to product. In one instance 52% of the activity was lost in 25 days and with another product the loss was 17% in 30 days. About a year was required for most of the activity to be lost.

A more detailed study was made of prothrombin preparation No. 491215. Large quantities were made available so that electrophoretic data could be obtained. Analysis of the product immediately after drying indicated that there was no change in activity. A sample was taken for electrophoresis at this time and the boundary patterns were like those obtained with other high quality prothrombin products. After 20 days 12% of the activity was gone, after 50 days 36%, and on the 124th day approximately half of the prothrombin activity was refractory to activation with calcium, thromboplastin and Ac-globulin. Some of the remaining dried material was then examined in the Tiselius apparatus. The boundary patterns were similar to the ones previously obtained with the same prothrombin immediately after it

had been dried. The mobility and the percentage composition were essentially the same. By these criteria, therefore, the inactive prothrombin could not be distinguished from the active material.

*Activation of altered prothrombin by autocatalysis.* If purified prothrombin is dissolved in a 25% solution of sodium citrate, it becomes activated by autocatalysis. Any thrombin added to such a solution initiates or accelerates the process. If, in addition, a small amount of 3-chloro-4,4'-diaminodiphenyl sulfone is added the yield of thrombin is maximal(6). This fact suggested the possibility that one might be able to obtain thrombin from the prothrombin which could not be activated with calcium, thromboplastin, and Ac-globulin. It has been repeatedly possible to do so. The details of one activation experiment are shown in Fig. 1. Prothrombin No. 491215 was used on the 124th day of storage at which time it had lost half its activity.

In the sodium citrate solution, containing 3-chloro-4,4'-diaminodiphenyl sulfone and 400 units of purified thrombin per cc, the thrombin activity lost during storage in the desiccator was completely recovered as thrombin. From one point of view this can be regarded as a 200% yield of thrombin. In some experiments with other products, yields of more than 1,000% have been obtained by this criterion. This simply means that the sodium citrate solution activated a prothrombin derivative which could not be activated with calcium, thromboplastin and Ac-globulin. It is an interesting fact that the prothrombin which could still be activated with "physiological" substances became refractory to them in the sodium citrate solution very early in the experiment. The activation then involved the production of thrombin almost entirely from inert prothrombin; that is, protein which could not be converted to thrombin with calcium, thromboplastin, and Ac-globulin under the conditions of the modified 2-stage analysis. About half had become inert in the desiccator during a period of 124 days

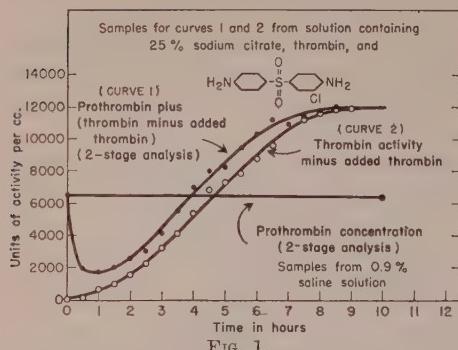


FIG. 1.

The purified prothrombin had lost 50% of its activity. A sample was dissolved in 25% sodium citrate solution. To this was added 400 units of thrombin per cc of solution and enough 3-chloro-4,4'-diaminodiphenyl sulfone to saturate the solution with respect to the sulfone. From this solution samples were taken for 2-stage prothrombin analysis and also for thrombin analysis.

and the other half in the sodium citrate solution within 30 minutes. Presumably the manner in which the prothrombin became inert did not influence the rate of transformation to thrombin in sodium citrate solutions. The question arises whether there were two kinds of altered prothrombin being activated simultaneously; however, it cannot be answered with the data now available.

**Discussion.** The prothrombin derivative produced by mixing prothrombin and thrombin in aqueous solutions has a lower electrophoretic mobility than the original prothrombin(1). The altered prothrombin produced after freeze drying has electrophoretic properties identical with fully active prothrombin. It must, therefore, be concluded that this paper describes a new derivative of pro-

thrombin which is different from the one produced through the action of thrombin. Both can, however, be activated to thrombin by autocatalysis in sodium citrate solution.

It is evident that one cannot expect to preserve prothrombin samples after they have been dried from the frozen state unless conditions can be found under which the activity will be retained. This is a major practical handicap because the freeze drying technic is convenient not only in the research laboratory but also in industry. Perhaps it is possible to dry prothrombin by using cold acetone. In quite a number of experiments it has been possible to do so without loss of activity. Furthermore, the acetone dried material seems to be stable; however, our experience covers a period of only one half year. It is, therefore, possible to say only that this procedure promises to be satisfactory.

**Summary.** Purified prothrombin can be dried from the frozen state without immediate loss of activity. Thereafter progressive loss of prothrombin activity occurs, characterized by refractivity to the action of calcium plus thromboplastin plus Ac-globulin. In about one year most of the prothrombin is altered and in addition some is insoluble in aqueous solution. Prothrombin altered as the result of the freeze drying technic can be activated autocatalytically in 25% sodium citrate solution. The altered prothrombin has essentially the same electrophoretic properties as purified prothrombin and represents a derivative of prothrombin not previously described.

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## Effect of Dibenamin on the Vascular Response of Rabbits to Typhoid Endotoxin. (18163)

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Menten and Manning(1) observed that injections of *E. coli* and *S. paratyphi* induced hyperglycemia in animals. Zeckwer and

Goodell(2) and later Evans and Zeckwer(3) confirmed these results and stated that hyperglycemia was due to the action of adrenalin.

Similar observations were reported by Boivin and Mesrobeanu(4).

We have shown(5) that intraperitoneal or intravenous injections of 1.5 to 3.0 ml of typhoid endotoxin, obtained by the Boivin method, killed rabbits weighing 2,000 to 2,500 g within a few hours, and that the initial symptom of intoxication was an intense peripheral vasoconstriction. This reaction commenced 5 to 15 minutes after the injection; it was followed by dyspnea, intense diarrhea, elevation of internal temperature, which lasted until the preagonal stage. If this phenomenon is due to the action of adrenalin or adrenaline-like substances, then it should be possible to modify or inhibit this vascular response by adrenolytic substances. Dibenamin (N-N dibenzyl-beta-chlorethylamine) was therefore selected for our study.<sup>†</sup>

*Materials and methods.* Albino rabbits weighing 2,000 to 2,500 g were used. The endotoxin was obtained from *Salmonella typhosa* by extraction of the organisms with trichloroacetic acid (Boivin and Mesrobeanu)(6). Five- to eight-tenths ml of this preparation, per kg of body weight, was injected intravenously. Six to 10 mg of Dibenamin per kg of weight was diluted to 10.0 ml in physiological saline, injected slowly (2 to 3 minutes) intravenously. These injections were made from 30 to 180 minutes following the administration of the endotoxin.

The cutaneous circulation was observed by

\* Holder of a special foreign fellowship from the Commonwealth Fund.

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2. Zeckwer, I. T., Goodell, H., *J. Exp. Med.*, 1925, v42, 43.

3. Evans, C. L., and Zeckwer, I. T., *Brit. J. Exp. Path.*, 1927, v8, 280.

4. Boivin, A., and Mesrobeanu, L., *C. R. Soc. Biol.*, 1934, v117, 273.

5. Boquet, P., Bovet, D., and Lehoul, Y., *C. R. Acad. Sci.*, 1947, v224, 1671; Boquet, P., Delaunay, A., Lehoul, Y., and Lebrun, J., *C. R. Acad. Sci.*, 1947, v224, 1671.

† Kindly supplied by the Smith, Kline and French Laboratories, Philadelphia, Pa.

6. Boivin, A., and Mesrobeanu, L., *C. R. Soc. Biol.*, 1933, v114, 307.

3 different technics: (1) observation of the small vessels in the ear by means of Clark's "preformed tissue" chamber; (2) direct observation of these vessels under the microscope(7); (3) an indirect method in which the temperature variations of the skin of the ear were taken with the aid of thermocouples. It has been established that these temperature variations are proportional to the blood supply in the skin and therefore to the intensity of the vasoconstriction. This method permits a graphic recording of the results and serves as a comparison with the other two technics.

*Results.* Intravenous injection of 1 m.l.d. of endotoxin changed markedly the rhythm of contraction and distension of small arteries. A long phase of contraction ensued so the arterioles and metarterioles were completely obliterated for several minutes. This was followed by short periods of relaxation. The process continued until the terminal stage of the intoxications (Curve 1). The final outcome was not changed by the administration of Dibenamin, although the intensity of diarrhea was markedly decreased. On the other hand, if the injection of endotoxin was preceded by intravenous administration of Dibenamin, the vasoconstriction was greatly modified (Curve 2). After a short period of contraction, the normal rhythm was temporarily re-established.

When administered after the injection of endotoxin, Dibenamin rapidly suppressed the contraction (Curve 3). It has been shown that denervated peripheral vessels are more sensitive to adrenalin or sympathin than the normal vessels(8); similarly we have demonstrated that denervated vessels of the rabbit's ear reacted to the endotoxin with greater intensity than the normal vessels(9). This intense contraction of the denervated vessels

7. Boquet, P., Lehoul, Y., Guichard, A., *Ann. Inst. Pasteur*, 1947, v73, 912.

8. Grant, R. T., *Heart*, 1930, v15, 257; Lecompte, Ph. M., *Amer. J. Physiol.*, 1942, v136, 669; Levinson, S. P., and Essex, H. E., *Proc. Soc. Exp. BIOL. AND MED.*, 1943, v52, 361; Essex, H. E., Herrick, J. F., Baldes, E. T., and Mann, F. C., *Am. J. Physiol.*, 1943, v139, 361.

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## CARBOHYDRATE EFFECT ON CHICK GROWTH

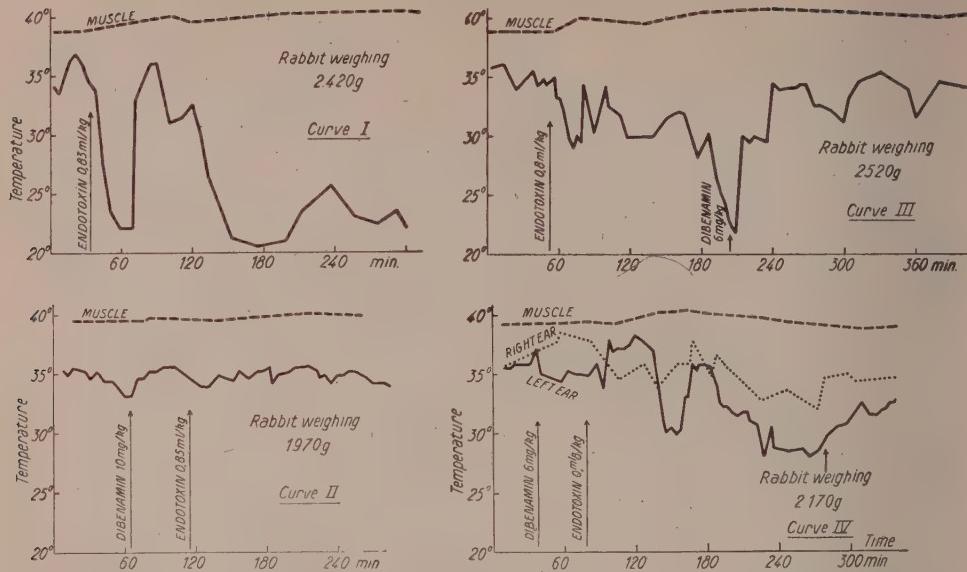


FIG. 1.

Effects of injections of dibenamin before and after the administration of typhoid endotoxin on the skin temperature of the rabbit's ear.

Curve 1—Skin temperature after an intravenous injection of typhoid endotoxin (1 m.l.d.).  
 Curve 2—Effect of an injection of dibenamin before the administration of the toxin.  
 Curve 3—Effect of an injection of dibenamin after the administration of the toxin.  
 Curve 4—Effect of 2 injections of dibenamin, one before and one after the administration of the toxin (the left ear has been denervated four days before the experiment).

In the 4 curves, the internal temperature is indicated by a dotted line.

can be decreased by previous administration of Dibenamin. However, to maintain this effect, it is necessary to follow with another injection of Dibenamin (3 mg per kg) (Curve 4).

*Summary and conclusions.* The injection *S. typhosa* endotoxin appears to stimulate the liberation of adrenalin or adrenalin-like sub-

stances, bringing about peripheral vasoconstriction. Dibenamin, a sympatho-adrenal blocking substance, antagonizes this effect.

We are indebted to Dr. E. R. Clark for his interest and continuous advices in our investigation.

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### Studies on the Effect of Different Carbohydrates on Chick Growth.\* (18164)

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Nutritional differences among sucrose, dextrin, lactose, and cellose have been reported by several workers. Elvehjem(1,2) has re-

viewed much of the work done on carbo-

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TABLE I. Effect of Supplements on Growth of Chicks Receiving Different Carbohydrates.

Treatment	4-week % wt gain (basal groups = 100%)*			
	Dextrin	Sucrose	Lactose	Cerelose
Basal	279 g = 100% (36)	206 g = 100% (36)	149 g = 100% (32)	259 g = 100% (12)
Folic acid deficient	35% (12)	22% (17)	53% (11)	—
Basal +				
0.1 µg vit. B <sub>12</sub> /day inj.	119 (12)	113 (12)	107 (12)	—
1.0 µg	110 (12)	118 (11)	134 (8)	—
0.6 µg vit. B <sub>12</sub> /100 g ration	113 (12)	—	127 (12)	—
reticulogen (1 U.S.P. unit/day inj.)	114 (12)	121 (12)	142 (9)	—
fish solubles (3%)	110 (12)	107 (12)	143 (11)	—
sulfasuxidine (2%)	100 (12)	81.5 (12)	86.5 (9)	—
sulfasuxidine (2%) + cellulose (10%)	88 (12)	86 (12)	74 (11)	—
cellulose (10%)	91 (12)	84 (12)	—	—
2 × B vitamins	98 (12)	100 (12)	—	—
Basal (50%) + 50% dextrin	—	117 (12)	174 (12)	—

\* Figures in parentheses indicate the number of chicks surviving. Those with numbers greater than 12 indicate that two or three experiments were run.

hydrate differences in experimental animals. Only a small amount of work has been done on the effect of different carbohydrates in rations for the chick. Luckey *et al.*(3) found that the folic acid requirement of the chick varies with the type of carbohydrate. Couch *et al.*(4) showed that the biotin content of eggs from hens receiving sucrose as the carbohydrate is much lower than that of eggs from hens on dextrin rations. Sarma *et al.*(5) presented differences in vitamin B<sub>6</sub> synthesis with different carbohydrates.

It is the purpose of this paper to compare the nutritional value of sucrose, dextrin, lactose, and cerelose and to study the effect of folic acid, vit. B<sub>12</sub>, and other growth factors on these carbohydrates as they affect chick growth.

**Experimental.** Straight run (New Hampshire ♂♂ X Single Comb White Leghorn ♀♀) cross bred chicks, which were the progeny of

1. Elvehjem, C. A., *J. Am. Dietet. Assoc.*, 1946, v22, 959.

2. Elvehjem, C. A., *Federation Proceedings*, 1948, v7, 410.

3. Luckey, T. D., Moore, P. R., Elvehjem, C. A., and Hart, E. B., *PROC. SOC. EXP. BIOL. AND MED.*, 1946, v62, 307.

4. Couch, J. R., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., *J. Nutrition*, 1948, v35, 57.

5. Sarma, P. S., Snell, E. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1946, v165, 55.

hens fed diet B-1 described previously(6) were used in all studies. The chicks were housed in electrically heated batteries with raised screen floors. Feed and water were supplied *ad libitum*. The chicks were wing-banded and weighed at 1 day of age. Weights were recorded at weekly intervals. Each group contained 12 birds. All chicks were divided into groups according to weight and immediately placed on test for four weeks. They all received a semi-purified ration containing carbohydrate 61 g, alcohol extracted casein 18 g, gelatin 10 g, salts V(7) 6 g, soybean oil 5 g, L-cystine 0.3 g, thiamine hydrochloride 0.3 mg, riboflavin 0.6 mg, nicotinic acid 5.0 mg, pyridoxine hydrochloride 0.4 mg, calcium pantothenate 2.0 mg, choline chloride 150 mg, biotin 0.03 mg, inositol 100 mg, 2-methyl-1,4-naphthoquinone 0.05 mg, *a*-tocopherol 0.3 mg, and folic acid 0.2 mg (except where indicated). Fortified haliver oil (60,000 U.S.P. units of vit. A, 6,000 U.S.P. units of vit. D<sub>3</sub> per g) was given by dropper (2 drops per bird per week). All supplements were added at the expense of the carbohydrate. Table I was compiled by cal-

6. Robblee, A. R., Nichol, C. A., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., *Poultry Sci.*, 1948, v27, 443.

7. Briggs, G. M., Jr., Luckey, T. D., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1943, v148, 163.

culating the per cent gain or retardation of each group relative to that group's basal control. The basal groups are shown as 100%, and the weight in grams is the average of the three experiments run.

*Results and discussion.* Table I shows that the chicks receiving the basal ration containing dextrin grew 73 g more than those receiving sucrose. The chicks receiving cerelose grew 53 g more than the sucrose-fed birds, and the birds receiving lactose grew 57 g less than the sucrose-fed birds. The feathering and general appearance of the birds receiving dextrin, cerelose, or sucrose were excellent. By the end of the first week, the chicks on the lactose diets developed severe diarrhea which lasted throughout the test period. Their general appearance was very poor, which may have been due partly to the fact that it was impossible to keep their cages clean and dry. These birds showed no decrease in food consumption, and they consumed about twice the amount of water consumed by the other birds, indicating the extent of the diarrhea.

When folic acid was omitted from the basal rations, there was a very significant decrease in growth in all cases. Although the birds receiving lactose showed the smallest percentage decrease, the birds receiving dextrin showed the best growth. This confirms work done by Luckey *et al.*(3) who showed that the chick has a smaller requirement for folic acid on rations containing dextrin than on rations containing sucrose.

When chicks receiving the basal rations were injected with a low level of vit. B<sub>12</sub>, slight responses were obtained with all carbohydrates tested. However, when a higher level of vit. B<sub>12</sub> was injected or fed orally, the response obtained with lactose was greater than the responses obtained with the other carbohydrates. Reticulogen and fish solubles also gave a better response with chicks on a lactose diet than with chicks on diets containing sucrose or dextrin. This may indicate that chicks on a lactose diet have a higher requirement for unknown growth factors which have been reported to be present in these preparations.

We have shown previously(8) that sulfasuxidine affects the growth of chicks as well as the liver storage and caecal content of folic acid. When sulfasuxidine was fed to chicks on different carbohydrates, the growth of the birds receiving sucrose and lactose was retarded in contrast to no effect in the case of the dextrin-fed birds. This indicates that the intestinal flora established on a ration containing dextrin as the carbohydrate constituent is affected in a different way or to a different extent by sulfasuxidine than the flora of chicks fed sucrose or lactose.

Since growth responses had been reported by Lepp *et al.*(9) with cellulose when 18% casein was fed, 10% cellulose was tried but gave a growth depression in all cases. A combination of sulfasuxidine and cellulose did not produce any response. Groups of chickens were fed twice the level of folic acid and other B vitamins. This was done to determine whether the difference in folic acid requirements on dextrin and sucrose diets, or intestinal synthesis of other known factors, was the cause of better growth with dextrin. No further growth responses were observed. In no case was it possible to obtain as good growth with chicks fed the sucrose ration as with chicks fed the dextrin ration.

It was interesting to find when diets with a carbohydrate consisting of 50% dextrin and either 50% sucrose or lactose were fed; the chicks exhibited significantly better growth. The response was especially striking in the lactose group in which a weight gain of 115 g and complete disappearance of the diarrhea were observed.

Johansson *et al.*(10) showed that the flora of hens on a dextrin diet contain 10 times the number of coliforms as the flora of hens on a sucrose plus biotin diet. It may be that 50% dextrin is enough to change the intestinal flora to the apparently more favorable condition and in the case of the lactose

8. Dietrich, L. S., Monson, W. J., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., in press.

9. Lepp, A., Harper, A. E., and Elvehjem, C. A., *Poultry Sci.*, 1949, v28, 372.

10. Johansson, K. R., Shapiro, S. K., and Sarles, W. B., *J. Bacteriol.*, 1947, v54, 35.

TABLE II. Excretion Time of Chicks as Affected by Different Carbohydrates.\*

Carbohydrate	2 wk.	4 wk.	4 wk.
	minutes	Group 1 minutes	Group 2 minutes
Lactose	77 ± 7	86 ± 14	78 ± 6
Sucrose	115 ± 22	133 ± 6	149 ± 17
Dextrin	140 ± 8	177 ± 5	181 ± 11

\* Includes stand. error.  $\sqrt{\frac{d^2}{n(n-1)}}$ .

group to also eliminate entirely the dehydration caused by lactose.

It was thought that a clearer understanding of the intestinal effects would be had by determining the time it takes for food to pass through the digestive tract of the chicken. At 2 and 4 weeks, 5 chicks per group were put in individual cages and given feed and water *ad libitum*. Each bird was given a number zero capsule filled with carmine 40, which is not absorbed by the intestine. The length of time taken for red to first appear in the feces was taken as the excretion time. The results are shown in Table II. The excretion time of the dextrin birds was 20% longer than the sucrose birds. These data lend support to the theory that carbohydrate differences may be explained on the basis of intestinal synthesis or efficiency of carbohydrate breakdown in the gut. It would seem

that dextrin allows more time for synthesis to take place, since chicks fed this ration retain the food longer. This extra time may allow additional synthesis of some unknown chick growth factor or factors.

**Summary.** When the rate of growth of chicks is used to measure the nutritional efficiency of carbohydrates they fall in the following order: dextrin, cerelose, sucrose, and lactose. Cellulose, sulfasuxidine, reticulogen, fish solubles, or vit. B<sub>12</sub> do not change the significant differences observed when these carbohydrates are fed. However, the lactose-fed birds do give the greatest response to reticulogen, fish solubles and the higher levels of vit. B<sub>12</sub>. The excretion times of chickens fed these different carbohydrates are in the following decreasing order: dextrin, sucrose, and lactose. The possibility of these nutritional differences being explained by the synthesis of known and/or unknown factors is discussed.

We are indebted to Merck and Co., Inc., Rahway, N. J., for crystalline vit. B<sub>12</sub>, and crystalline vitamins; to the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., for synthetic folic acid; to Abbott Laboratories, North Chicago, Ill., for haliver oil; to Wilson and Co., Inc., Chicago, Ill., for gelatin; and to E. I. duPont de Nemours and Co., Inc., New Brunswick, N. J., for crystalline vit. D<sub>8</sub>.

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## Experimental Production of the L. E. Phenomenon in the Skin of Man.\* (18165)

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The L.E. cell, first described by Hargraves, Richmond and Morton(1), was originally observed in incubated marrow aspirates of pa-

\* Presented before the Joint Meeting of the North Central Region College of American Pathologists and the Michigan Pathological Society, Detroit, Mich., May 13, 1950.

1. Hargraves, M., Richmond, H., and Morton, R., *Proc. Staff Meet. Mayo Clinic*, 1948, v23, 25.

tients suffering with acute disseminated lupus erythematosus. Sundberg and Lick(2) found occasional L.E. cells in the peripheral blood in acute disseminated lupus erythematosus although again leukocytic concentration in the test tube was employed. Haserick and Bortz

2. Sundberg, R. D., and Lick, N., *J. Invest. Derm.*, 1949, v12, 83.

(3) and Hargraves(4) next produced L.E. cells *in vitro* by utilizing control marrows incubated with only the plasma of patients acutely ill with disseminated lupus erythematosus. This report describes the production of the L.E. cell phenomenon (Fig. 1B, 3, 4) in the skin of normal human volunteers following inoculation of experimental windows with the plasma of a patient with acute disseminated lupus erythematosus.

*Materials and methods.* Five experimental windows were prepared in 2 human male volunteers. The technic of human window preparations has been described in detail elsewhere(5-8). Briefly, the epithelium was slowly scraped away with a sterile scalpel over a small area in the forearm until the papillary layer of the corium was exposed. In the present experiments a small drop of diphtheria toxoid or egg-white was next placed on the lesion to increase the toxicity of the local area. The lesion was then covered with a sterile glass cover-slip which in turn was surmounted

by a sterile square of cardboard and both were covered with surgical adhesive tape. Within an hour or 2 the cells of the inflammatory exudate migrated to the under-surface of the cover-slip. At timed intervals the cover-slip was removed from the lesion, air-dried and stained with May Grünwald-Giemsa stain. The lesion was again immediately covered by a second cover-slip. In this way samples of the cellular exudate were obtained from the same lesion every few hours throughout the simple inflammatory cycle. Two to 3 drops of the L.E. factor plasma were added to the lesions at from 5.5 to 9.5 hours after the initiation of the lesions. Five control experiments were performed in which 4 lesions were inoculated with diphtheria toxoid without the addition of the L.E. factor. In the final control experiment the human volunteer was inoculated with diphtheria toxoid, followed at suitable intervals by inoculation of normal human plasma.

*Experimental results.* Lesions 1 and 2 were studied in the first human male volunteer; he was Schick negative. In lesion 1 the original inoculant was diphtheria toxoid. Cover-slips were removed at 3.5, 5.5, 9, 11, 14 and 23 hours. The L.E. plasma was introduced at the 9th hour of inflammation, and the L.E. phenomenon was observed in the next preparation, *i.e.* the 11th hour of inflammation or 2 hours after the addition of the L.E. factor to the lesion. In lesion 2 the original inoculant was egg-white. Cover-slips were removed at 3.5, 5.5, 7.5, 11.5, 14 and 23 hours. The L.E. plasma was introduced at 5.5 hours of inflammation and the L.E. phenomenon was observed in the next preparation, *i.e.* 7.5 hours of inflammation or 2 hours after the addition of the L.E. factor to the lesion.

Lesions 3, 4 and 5 and control lesions 9 and 10 were studied in a second human male volunteer who was Schick positive. The original inoculants in these five experiments were the same, diphtheria toxoid. In lesion 3 cover-slips were removed at 3, 6, 8, 8.75, 9.5 and 11 hours. The L.E. plasma was introduced at 8 hours of inflammation and the next preparation, *i.e.* at 8.75 hours of inflammation or 45 minutes later failed to show a

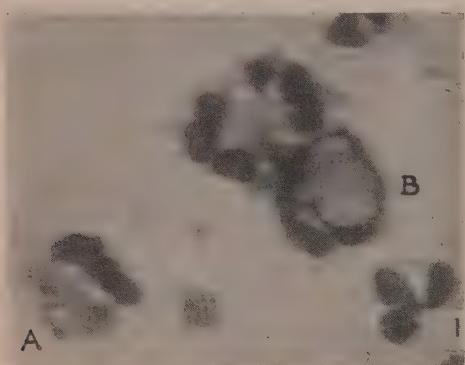


FIG. 1.

Lesion 1. Experimental. (A) Partial lysis of nuclear lobes forming 2 "ring lobes." (B) L.E. cell. 11 hr of inflammation in man.  $\times 1400$ .

3. Haserick, J. R., and Bortz, D. W., *J. Invest. Derm.*, 1949, v13, 47.
4. Hargraves, M. M., *Proc. Staff Meet. Mayo Clinic*, 1949, v24, 234.
5. Rebuck, J. W., Thesis University of Minnesota, 1947.
6. Rebuck, J. W., and Woods, H. L., *Blood*, 1948, v3, 175.
7. Rebuck, J. W., and Monaghan, E. A., *Fed. Proc.*, 1948, v7, 277.
8. Rebuck, J. W., *Anat. Rec.*, 1949, v103, 497.



FIG. 2.

Lesion 4. Experimental. Free partially lysed nuclear lobes amid granular and filamentous nuclear debris. 13 hr of inflammation in man.  $\times 2000$ .



FIG. 3.

Lesion 2. Experimental. L.E. cell with 2 additional small ingested nuclear bodies. 7.5 hr of inflammation in man.  $\times 2000$ .

FIG. 4.

Lesion 1. Experimental. L.E. cell. 11 hr of inflammation in man.  $\times 1400$ .

clear-cut L.E. phenomenon. Lesions 4 and 5 were then modified as follows: In lesion 4 cover-slips were removed at 4, 7, 9.5, 11.75 and 13 hours. The L.E. plasma was introduced at 9.5 hours of inflammation, and the next preparation at 11.75 hours again failed to show a clear-cut L.E. phenomenon; thereupon two more drops of the L.E. plasma were added to the same lesion and the next preparation, *i.e.* at 13 hours of inflammation or 1.25 hours after the second application of the L.E. factor presented the L.E. phenomenon. In lesion 5, cover-slips were removed at 4, 6.75, 9.5, 11.25, 12 and 13 hours. The

L.E. plasma was introduced at 9.5 hours of inflammation and the next preparation at 11.25 hours again failed to show a clear-cut L.E. phenomenon; thereupon additional L.E. plasma was applied to the same lesion and the next preparation, *i.e.* at 12 hours of inflammation or 45 minutes after the second application of the L.E. factor presented the L.E. phenomenon.

Lesions 6 to 10 comprised the control lesions; in lesions 6 to 9 cover-slips were removed from the second to the twenty-fourth hours of inflammation at intervals of a few hours. In lesions 6 to 9 diphtheria toxoid alone served as the inoculant. In control

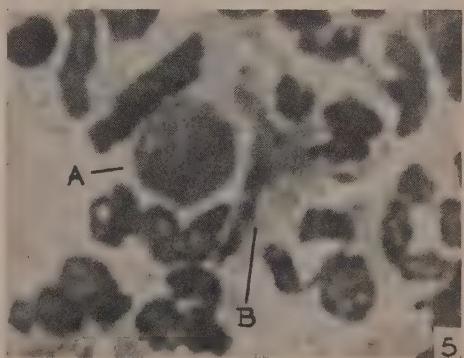


FIG. 5.

Lesion 1. Experimental. (A) Free partially lysed nucleus. (B) Nuclear debris. Note "rosette" of surrounding neutrophilic leukocytes. 11 hr of inflammation in man.  $\times 1700$ .

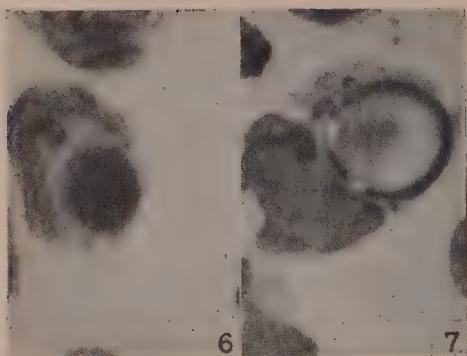


FIG. 6.

Lesion 9. Control. Lymphocyte with ingested nuclear mass. 12 hr of inflammation in man.  $\times 2000$ .

FIG. 7.

Lesion 9. Control. Histiocyte (macrophage) with ingested nuclear mass. 16.5 hr of inflammation in man.  $\times 20000$ .

lesion 10 in addition at 7 and 9 hours of inflammation normal heparinized plasma (in place of the L.E. plasma of the experimental lesions) was added to the lesion from which cover-slips were removed at 2.5, 5, 7, 9, 11.75 and 13.25 hours.

In the experimental lesions 1, 2, 4 and 5 then the L.E. phenomenon appeared in the inflammatory exudate in from 2 to 3.5 hours after introduction of the L.E. factor plasma. Because of the dynamics of the window technic the progressing cytolytic processes were as prominent as were the L.E. cells themselves. In the original description(1) the L.E. cell was usually a mature neutrophilic polymorphonuclear leukocyte which had either ingested free exogenous nuclear masses of homogenous, purple chromatin material or had suffered partial autolysis of its own nuclear lobes. The window technic afforded a fuller exposition of the cytolytic phenomenon than previous *in vitro* technics have offered.

The first structural changes observed in the exudative neutrophilic leukocytes exposed to the necrotizing factor of the L.E. plasma were apparent in the neutrophilic granules, the cell body, and polymorphous nucleus. The changes in the granules were non-specific swelling and increased basophilia together with clumping and loss through cytoplasmic

budding. Changes in the cell body were likewise largely non-specific, its disintegration was accompanied by vacuolation and cytoplasmic budding or shedding. The nucleus was relatively more resistant to the necrotizing factor and nuclear lysis proceeded in stages. Unlike the pyknosis of ordinary neutrophilic degeneration in the controls which was accomplished by shrinkage and clumping of nuclear lobes, leading to formation of rounded hyperchromatic nuclear remnants, karyolysis of neutrophils in the experimental windows was initiated by early swelling of one or more of the individual nuclear lobes. Swelling of the individual lobes was soon followed by marked changes in the chromatin-parachromatin pattern. The ordinary coarse, chromatin blocks formed a smooth homogenous mixture with the paler parachromatin; gradual effacement of the lobar chromatin pattern than resulted. Commonly such swollen, homogenous waxy nuclear masses were set free into the exudative fluids at this stage of karyolysis and ingested by still viable neutrophilic leukocytes, thus producing one form of the L.E. cell. Not too infrequently lobar lysis proceeded within the originally affected nucleus. Further lysis was signaled by lobar imbibition of fluid which not only brought about further swelling but also resulted in progressively paler staining of the affected structures. The central portion of the lobe became vacuolar, the chromatic mixture was pushed to the periphery with the formation of ring-like lobes, (Fig. 1A) a finding previously described as "ring nuclei" by Ash and Spitz(9) in the polymorphonuclear leukocytes of stools in the Shigella infections.

The second group of structural changes observed were associated with dissolution of the affected neutrophils freeing the affected lobes undergoing varying degrees of lysis. Thus free, partially lysed nuclei and/or nuclear lobes were scattered among the exudative cells (Fig. 2). The possibility that small portions of the toxic cytoplasm were occasionally still attached to the lysing nuclear material

could not be ruled out. The relatively intact neutrophilic leukocytes were apparently attracted to the nuclear and lobar bodies.

The third stage in the development of the ultimate inclusion phenomenon was the ingestion of the partially lysed swollen lobes and nuclei presenting partial or complete homogenization of their chromatin and other nuclear materials (Fig. 1B, 3, 4).

The last stage in the development of the inclusion phenomenon was the further digestion by the relatively intact neutrophils of the ingested lobar and nuclear material. The phagocytosed chromatic portions became progressively paler until affinity for the basic dyes was finally lost. There remained a large empty vacuole with a faint lavender pellicle marking the site of the former nuclear or lobar membrane.

Variants were noted in the interplay of the 4 stages. Nuclear lysis and degradation might proceed to the very late stages while the nuclei or their lobes were still free in the exudate. Phagocytosis at this later stage resulted in phagocytosis of "ring lobes" or "ring nuclear remnants." As Hargraves *et al.*(1) originally suggested, there was some evidence that all four stages of karyolysis could go on to completion within the original neutrophilic leukocyte, the individual lobes differing as to their susceptibility to the L.E. necrotizing factor. Thus the lobes were observed in different stages of lysis within a single cell (Fig. 1A). Homogenization of the nuclear material at times preceded the swelling of the lobes. Occasionally the nuclear material was found in the form of numerous small, partially lysed nuclear fragments and bodies much smaller than individual lobes pointing to fragmentation of the typical L.E. bodies prior to (Fig. 2) or subsequent to ingestion (Fig. 3). Host cells may fare quite well (Fig. 3); at other times they too seemed to be deleteriously and rapidly affected either by the necrotizing factor of their general environment or by the fact of ingestion of an affected nuclear lobe (Figs. 1B, 4).

"Rosettes" or polymorphonuclear leukocytes clumped around nuclei (Fig. 5A) or amorphous masses (Fig. 5B) of nuclear debris

as described by Haserick and Bortz(3) were also present *in vivo*. However, the masses appeared to be purple and not acidophilic as originally described. The central mass may resemble (Fig. 2) the stringy desoxyribose nucleoprotein studied by Sherry, Tillett and Christensen(10) in purulent pleural exudates.

We were not able to find L.E. cells in the numerous windows obtained from the control lesions. In lesion 9 in which diphtheria toxoid alone served as the inoculant at the 12th hour of inflammation, the lymphocytes were at times phagocytic for nuclear debris (Fig. 6). At 16.5 hours of inflammation in the same lesion an occasional small histiocyte (macrophage) had ingested a round nuclear remnant (Fig. 7). In this instance there had occurred some intracellular homogenization of the phagocytosed nuclear material. Although uncommon in the control material, such a finding as illustrated in Fig. 7 depicting as it does the ability of the histiocyte to bring about intracellular homogenization of phagocytosed nuclear material may afford a clue as to the source of the enzymes responsible for the nucleic acid depolymerization, a depolymerization claimed for systemic lupus erythematosus by Klemperer and his associates(11). Such ingested nuclear materials were never observed within the cell-bodies of neutrophilic leukocytes in the control lesions and conversely histiocytes rarely became the host L.E. cells in the experimental lesions.

The cover-slip preparations obtained subsequent to the production of the L.E. phenomenon contained few or no L.E. cells. In the 5 months since these experiments were completed no evidence of acute disseminated lupus erythematosus has been noted in the two human volunteers. We advise against repetition of these experiments in man until such a time as it is possible to utilize active necrotizing L.E. factor plasma known to be free of any contamination with the virus of homologous serum hepatitis. We are indebted to Dr. R. R. Margulis for his generous help

10. Sherry, S., Tillett, W. S., and Christensen, I. R., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 179.

11. Klemperer, P., Gueft, B., and Lee, S., J. Mt. Sinai Hosp., 1949, v16, 61.

proffered in these experiments.

*Summary and conclusions.* The L.E. cell phenomenon has been produced experimentally in the skin of two normal human volunteers following inoculation of windows with the plasma of a patient with acute disseminated lupus erythematosus. *In vivo*, under the conditions of our experiments, the neutrophilic L.E. cell usually developed as the result of ingestion of other neutrophilic nuclear lobes or nuclei which had undergone a previous partial peculiar lysis. Less commonly the L.E. cell may represent the originally affected neutrophilic

leukocyte in which lobar lysis is out of step in the individual nuclear lobes. The experiments described should provide a means of testing the proposed identity of the L.E. cells and the "hematoxylin staining bodies" of the diseased tissues in acute disseminated lupus erythematosus, an identity recently suggested by several workers(11,12).

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### The Antipyretic Effect of Cortisone.\* (18166)

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(Introduced by R. F. Loeb.)

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In various febrile illnesses, clinical improvement following the use of adrenocorticotropic hormone (ACTH) or cortisone is usually associated with a decline in temperature to normal or near-normal levels(1). The decline occurs together with a striking diminution in the clinical and laboratory evidences of inflammatory activity. There have been several reports illustrating an antipyretic effect of ACTH without appreciable alteration in the course of the underlying disease. Thus, in pulmonary tuberculosis(2), pneumo-

coccal pneumonia(3) and subacute bacterial endocarditis(4), the administration of ACTH was followed by apparent clinical well-being and rapid defervescence of fever, but cultures of the blood or sputum continued to be positive. Indeed, in one instance extension of the tuberculous process has been noted to occur during ACTH administration. In view of these observations, it became of interest to determine whether an antipyretic effect of cortisone could be experimentally demonstrated.

*Exp. I.* Thirty chinchilla rabbits of about 2 kg body weight were divided into two groups of equal number. Each animal of one group received an initial injection of 10 mg of cortisone acetate† intramuscularly, fol-

\* This work is supported in part by the Helen Hay Whitney Foundation and the Masonic Foundation for Medical Research and Human Welfare.

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† Obtained from Merck and Company on recommendation of the Committee on Cortisone of the National Academy of Sciences, through funds allocated by the U. S. Public Health Service.

TABLE I. Effect of Cortisone on the Febrile Response of Rabbits to Pneumococcus Vaccine.

	1. (Day 3)		2. (Day 10)		3. (Day 12)	
	Control	Cortisone	Control	Cortisone	Control	Cortisone
No. of animals	15	15	12	13	10	5*
						8
						13†
Mean temp. °F before pyrogen	—	—	102.4	102.0	102.0	102.3*
Mean temp. °F 2 hr after pyrogen	104.3	102.9	105.1	103.8	104.5	101.4
						101.8†
Mean temp. increment ± stand. error of mean	2.3 ± .25§	0.9 ± .15§	2.7 ± .17	1.8 ± .20	2.5 ± .18	1.2 ± .25*
Diff. between means of control and cortisone-treated groups divided by stand. error of this diff.	4.8		3.6			4.1*
						2.8

All animals received 2 cc of pneumococcus polyvalent vaccine on Day 1, and 4 cc on Days 10 and 12. Cortisone-treated animals received 2.5 mg a day except as indicated.

\* 10 mg a day for 3 days.

† Mean temp. of entire group, including animals receiving both 10 mg and 2.5 mg cortisone.

‡ Highly significant when the ratio is greater than 2.6.

§ For convenience of presentation, 102°F was used as the baseline temperature in this part.

lowed thereafter by a daily injection of 2.5 mg. On the third day of cortisone administration, both groups of animals received 2 cc of a polyvalent pneumococcal vaccine† intravenously. Rectal temperatures were found to reach a peak about two hours after the injection and to approach baseline in seven hours. Subsequently, eight and ten days later, after continued cortisone administration to the treated group of animals, 4 cc of pneumococcal vaccine was given intravenously to both groups and the rectal temperatures were again recorded. Therefore, the first febrile response was initiated after three days of cortisone administration, and the last one after twelve days of cortisone to the same animals. Five of the treated group of animals were given an increased amount of cortisone (10 mg per day) for the three days preceding the last injection of pneumococcus vaccine.

The febrile responses of the rabbits are presented in Table I. The temperatures are recorded in degrees Fahrenheit, less 100°, for

convenience. It is evident that on each of the three occasions studied, the average febrile response of the cortisone-treated animals was 1.2° to 1.5° less than the control animals two hours after injection of the vaccine. These differences, although small, are highly significant statistically as indicated in the table. The differences between the mean temperatures of the control and cortisone-treated groups divided by the standard error of these differences range from 2.8 to 4.8. When the ratio is greater than 2.6, the probability of exceeding the difference by chance factors alone is less than 1 in 100. As further indicated in part 3 of Table I, the animals receiving 10 mg of cortisone for the last three days of the experiment showed a smaller increment in temperature from their baseline temperature than did the animals maintained on 2.5 mg daily, namely, 1.2° ± .25 as opposed to 1.7° ± .22.

*Exp. II.* A standardized pyrogenic assay procedure was employed (5). Two groups of 9 rabbits each (A and B) were given a pseud-

† Obtained from Dr. F. Kauffmann of the State Serum Institute, Copenhagen for use in experiments to be reported with Dr. M. Bjørneboe and Dr. H. C. Stoerk. (*J. Exp. Med.*, 1950, v93, No. 1).

§ We are indebted to Dr. J. Fertig, Professor of Biostatistics, Columbia University School of Public Health, for his help and criticism.

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TABLE II. Effect of Cortisone on the Febrile Response of Rabbits to Pseudomonas Vaccine.

Group	No. of rabbits	Procedure	Mean temp. rise in °F
A	9	Initial dose of pyrogen	2.5 ± .23
		2nd dose of pyrogen + cortisone*	1.3 ± .14
B	9	Initial dose of pyrogen	2.0 ± .20
		2nd dose of pyrogen + saline†	2.0 ± .18

\* Received 5 mg cortisone subcut. for 3 days prior to the second dose of pyrogen.

† Received 1 ml 0.5% pyrogen-free saline for 3 days prior to the second dose of pyrogen.

An interval of 2 mo. elapsed between the 1st and 2nd doses of pyrogen.

domonas pyrogenic vaccine and their febrile response was recorded. Two months later the pyrogen was administered for the second time. The second febrile response could be expected to reproduce the first(5). However, on this second occasion, group A was pretreated for three days with 5 mg of cortisone (saline suspension 5 mg/ml/rabbit subcutaneously) while group B received subcutaneously 1 ml of a pyrogen-free 0.5% saline solution for three days prior to the administration of the pyrogen. Rectal temperatures were recorded. As is evident from Table II, the animals of group B reacted with striking conformity to their pattern of two months previously. The cortisone-treated group A, although previously found to have a slightly higher average febrile response than group B, manifested much less of a rise on this occasion. This result is statistically significant and in keeping with the observations in Exp. I.

**Discussion.** It is apparent that cortisone exerts a moderate antipyretic action. The quantitative aspects of this action have not

been investigated. It seems likely, however, that the degree of antipyretic effect is related to the dose of cortisone administered. This is suggested by the fact that the animals receiving the larger dose of cortisone showed a smaller febrile response to the same dose of pyrogen.

Antipyretic drugs may exert their effects by a peripheral action on the tissues or a central action on the thermo-regulator centers of the hypothalamus. There is increasing evidence that cortisone alters and often inhibits the inflammatory reaction of tissues to injury(6), although this may not always occur(7). Fever as a sequel to inflammation is thought to result from the products of the inflammatory exudate(8), and inhibition of the latter may be expected to decrease the febrile response. In addition, cortisone is known to act upon the central nervous system as evidenced by the electroencephalographic changes and psychic disturbances(9) noted in treated patients. It would seem possible that both an anti-inflammatory and central action of cortisone may be responsible for the antipyretic effect.

**Summary.** In a controlled study, cortisone has been found to be antipyretic in rabbits given pneumococcal vaccine and a pseudomonas pyrogen. The mechanism for this effect is not known.

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## Sex Difference in the Effect of Progesterone on Body Weight of Mice. (18167)

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In a number of species the female is normally smaller than the male. In the rat ovariectomy results in an increased body weight, to a level approaching that of the male. In the male, however, castration early in life results in no change or in a reduced body weight as compared to non-castrate controls(1-4). The effect of ovariectomy on body weight has been attributed to an elimination of the inhibiting effect of estrogen on body weight(4). The growth inhibiting effect of estrogen in a number of species has long been known. More recently progesterone has been reported to increase the body weight of rats(4-6).

During the course of an experiment designed to determine the effect of long continued progesterone administration on mammary tumor incidence of mice, a sex difference was observed in the effect of progesterone on body weight.

*Methods.* The mice used were first generation male and female AC<sub>2</sub> hybrids (C<sub>3</sub>H ♀ x A ♂) and male AB<sub>2</sub> hybrids (CBA ♀ x A ♂). The female AC<sub>2</sub> mice were maintained as virgins and divided into two littermate controlled groups of 60 mice each, of ages ranging from 2 to 5 months at the start of treatment. One group received a 14 ± 1 mg pellet of progesterone subcutaneously every 28 days until death or sacrifice when death seemed imminent. The other group received no treatment.

\* Fellow of the Jane Coffin Childs Memorial Fund for Medical Research. This investigation has been aided by a grant from The Jane Coffin Childs Memorial Fund for Medical Research.

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The considerably fewer animals in the progesterone treated group after 15 months of treatment (Table I) is the result of an earlier appearance of mammary tumors in this group. Weights of tumor bearing animals were not recorded. The male mice of each of the two stocks were divided into 8 groups, 4 intact and 4 castrated groups, each subdivided as follows: (a) untreated controls, (b) estrogen treated, (c) progesterone treated, (d) estrogen and progesterone treated. Estrogen treatment consisted of a 6 to 8 mg subcutaneous pellet composed of 25% diethylstilbestrol and 75% cholesterol. Such pellets are generally effective throughout the lifetime of a mouse. Progesterone treatment was the same as for the females. Castration was performed 1 to 3 days prior to the start of the experiment. The age of the mice used ranged from 1 to 5½ months at the start as indicated in Tables I to III. The oldest male mice at the time the experiment was set up were used as untreated controls in order to have the treated males as young as possible at the initiation of treatment. The reduction in number of mice in the estrogen treated groups with the passage of time is the result of mammary tumor development, and of the various other harmful effects of estrogen (bladder distension, hydronephrosis, scrotal hernia, etc.) All mice received Purina Fox Chow and water *ad libitum*.

Because the experiment was not designed with body weight changes in mind, the original body weights of the various groups were not obtained at the time treatment was started. Since the females were littermate controlled, it can safely be assumed that the initial body weights of the two groups were very similar. Among the males, the intact and castrated mice in each group were littermate controlled. Among the three classes of hormone treated males, the ages of the mice used were uniformly distributed within the age ranges indicated in Tables II and III. Differences un-

## PROGESTERONE EFFECT ON BODY WEIGHT

TABLE I. Body Weights of Progesterone Treated Intact Virgin Female Mice ( $AC_2$ ) and Their Untreated Littermate Controls.

	Age at start (mo.)	No. at start	5 mo.			Duration of treatment			15½ mo.		
			Body wt (g)	Age (mo.)	No.	Body wt (g)	Age (mo.)	No.	Body wt (g)	Age (mo.)	No.
Untreated controls	2-5	60	28.2	7-12	59	28.9	9-14	59	32.9	17-20	51
Progesterone treated	2-5	60	34.5	7-12	58	35.6	9-14	56	39.9	17-20	19

TABLE II. Body Weights of  $AC_2$  Male Mice Receiving Estrogen and Progesterone Treatment, Alone and in Combination.

	Age at start (mo.)	No. at start	Duration of treatment			7 mo.			15½ mo.		
			Body wt (g)	Age (mo.)	No.	Body wt (g)	Age (mo.)	No.	Body wt (g)	Age (mo.)	No.
Untreated controls	Intact	4½-5	14	38.6	11½-12	14	38.2	20-20½	14		
	Castrated	4½-5	13	40.0	11½-12	13	40.6	20-20½	13		
Progesterone treated	Intact	1½-4½	16	38.5	8½-11½	16	39.0	17-20	15		
	Castrated	1½-4½	17	38.3	8½-11½	17	37.1	17-20	17		
Estrogen + Progesterone treated	Intact	1-5	15	33.7	8-12	15	36.8	16½-20½	4		
	Castrated	1-5	15	31.9	8-12	15	33.1	16½-20½	8		
Estrogen treated	Intact	1-5	16	28.9	8-12	12	33.6	16½-20½	2		
	Castrated	1-5	16	28.4	8-12	14	27.4	16½-20½	2		

TABLE III. Body Weights of  $AB_2$  Male Mice Receiving Estrogen and Progesterone Treatment, Alone and in Combination.

	Age at start (mo.)	No. at start	Duration of treatment			8 mo.			16½ mo.		
			Body wt (g)	Age (mo.)	No.	Body wt (g)	Age (mo.)	No.	Body wt (g)	Age (mo.)	No.
Untreated controls	Intact	5½	13	39.1	13½	13	40.6	22	13		
	Castrated	5½	12	39.1	13½	11	37.7	22	10		
Progesterone treated	Intact	4-5	13	38.1	12-13	13	37.5	20½-21½	13		
	Castrated	4-5	14	37.4	12-13	14	37.6	20½-21½	11		
Estrogen + Progesterone treated	Intact	4-5	12	34.1	12-13	9	35.7	20½-21½	5		
	Castrated	4-5	13	32.9	12-13	12	31.9	20½-21½	3		
Estrogen treated	Intact	4-4½	18	30.8	12-12½	16	37.2	20½-21	4		
	Castrated	4-4½	18	30.0	12-12½	17	31.9	20½-21	7		

doubtedly existed between the original weights of the older untreated males and the younger treated males, particularly in the  $AC_2$  mice. This difference, however, is minimized by the long-term nature of the experiment, such that one is dealing eventually with maximum body weights attainable on each regime. Since the mice are  $F_1$  hybrids of inbred strains their growth potential should be highly comparable.

*Results.* Several months after treatment was

started, it became visibly apparent that the progesterone treated virgin females had attained a considerably larger body size than their untreated littermates. At the first weighing, after 5 months of treatment, the progesterone treated females averaged 6.3 grams (22%) heavier than their littermate controls. This prompted the recording of weights among the male mice also, with the surprising finding of no increase in body

weight of the progesterone treated males over the controls, after as long as 16½ months of treatment (Tables II and III). A protective action of progesterone against the body weight suppressing effect of estrogen is, however, apparent in all but the 16½ month weights of the AB<sub>2</sub> males. The lack of effect among the reduced numbers of AB<sub>2</sub> mice at this time may be the result of (a) selective mortality of the mice more susceptible to the harmful effects of estrogen, (b) bladder distension with urine retention in the surviving estrogen treated mice.

**Discussion.** The difference in the effect of progesterone on body weight of the male and female mouse suggests that the action of progesterone on body weight is not a direct anabolic effect, but rather an indirect effect dependent upon some mechanism present or possible in the female mouse but not in the male mouse. In this regard it is interesting to speculate that the mechanism of action of progesterone on body weight of the female mouse may be to a large extent identical with that of ovariectomy. Progesterone is known to suppress FSH secretion, rendering the ovary non-cyclic and reducing estrogen secretion (7-9). The suppression of the secretion of growth inhibiting estrogen may be largely responsible for the greater growth of the progesterone treated intact female. To this may be added the above mentioned effect of progesterone in partially protecting the animal against the growth inhibiting action of any estrogen that may be secreted. Vaginal smears taken on the control and progesterone treated females confirmed the effectiveness of progesterone in suppressing ovarian activity and/or over-riding the effect of estrogen on the vagina under the conditions of this experiment. Vaginal smears taken 5 and 15 months after initiation of treatment showed

no positives among the progesterone treated females (260 smears) while simultaneous smears (263) among the controls averaged 23% positive. Opposed to such an interpretation of the mechanism of action of progesterone on body weight is the possibility that in the rat progesterone may increase the body weight of males or ovariectomized females. The data in this regard, however, are conflicting and inconclusive. In groups of 6 rats receiving 2 mg of progesterone daily for 3 weeks, both the males and females gained more than their respective controls. The effect was greater in the females than in the males(5). In groups of 6 rats receiving 10 mg of progesterone daily for 10 days, young castrated progesterone-treated males gained the same as their controls, while old castrated progesterone treated males gained less than their controls(10). In groups of 2 rats receiving 5 mg of progesterone daily for 9 weeks the treated rats, both male and female, gained more than their controls. However, the extent of the difference was not statistically significant(6). In ovariectomized female rats receiving .05 mg of progesterone daily for 3 months, three large treated rats gained more than their untreated controls while 4 young treated rats gained the same as their controls(4). The need for further observations on possible differences in the action of progesterone on the body weight of the male versus the female and of the intact versus the ovariectomized female is indicated.

**Summary.** Virgin female mice receiving long term progesterone treatment attained an appreciably greater body weight than their untreated littermate controls. Male mice, both intact and castrate, receiving the same progesterone treatment failed to attain a greater body weight than their untreated controls. Progesterone exerted a protective action against the body weight suppressing effect of estrogen.

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## Electron-Microscopic Examination of Human Milk Particularly from Women Having Family Record of Breast Cancer.\* (18168)

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Mouse mammary carcinoma is caused by a filterable, thermolabile agent, which is transmitted from one generation to another through the milk of nursing females(1-3). Newly born female mice which ingest the pathogenic agent with the milk of their mothers remain in good health until they reach middle age; at that time the agent becomes activated(4), causing the development of mammary carcinomas. Young, nursing female mice which carry the tumor agent, and are earmarked to develop mammary carcinomas later in life, show therefore no symptoms of tumors, and appear to be in perfect health at the time they nurse their offspring; yet they discharge the tumor agent in their milk. Only their family record, if available, and particularly if it extends over the period of several preceding generations, may reveal a history of mammary carcinomas in some of the female ancestors on the mothers' side, indicating thereby that such nursing mice may themselves be carriers of the mammary carcinoma agent.

Recently, small spherical particles, having a diameter varying from 20 to 200  $\text{m}\mu$ , have been visualized with the aid of an electron-microscope in samples of milk obtained from nursing mice known to carry the mammary

carcinoma agent(5,6). Similar spherical particles have also been found in tumor cells(7), or in tumor cell extracts(6,8) prepared from either spontaneous, or transplanted mouse mammary carcinomas. Electron microscopic examination of milk(5,6) from mice of families free from spontaneous mammary carcinomas, as well as that of extracts(6,8) prepared from normal mammary glands of such mice, showed only small quantities of spherical particles in some of the samples(6,8), or none at all(5). The true nature of the spherical particles revealed with the electron microscope in the milk of nursing mice known to carry the mammary tumor agent remains yet to be determined, although some investigators are already inclined to feel that these particles actually represent the mouse mammary carcinoma virus(5). Since the possibility is at hand that human breast cancer may also be caused by factors similar to those responsible for the development of mammary carcinoma in mice(4,9), a series of preliminary experiments, reported in this study, has been carried out, with the purpose of determining whether spherical particles, similar to those found in mouse milk, would, perhaps, also be found, with the aid of an electron microscope, in samples of human milk, particularly in those obtained from women having a family record of breast cancer.

*Materials. Human Milk Samples.* Young, healthy women who had delivered 3 to 7 days

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previously, and were nursing at the time, were interviewed as to the occurrence of malignant tumors in members of their respective families. Two groups of women were selected as donors, as follows:

Group A. Ten milk samples were collected from nursing, healthy women, whose sisters, mothers, or grandmothers, had breast cancer.

Group B. Thirty-two control samples were collected from nursing, healthy women, with a family record apparently free from malignant tumors for 2 preceding generations.

*Preparation of Milk Samples for Electron Microscopy.* The milk samples were in most instances processed immediately; in 2 instances only, the samples, having been obtained over the weekend, were stored in a refrigerator at 7°C for a few days prior to processing. Several methods have been successively tried in the preparation of the milk samples for electron microscopy. At first, encouraged by the apparently satisfactory results obtained by Graff(5) and his colleagues in processing mouse milk samples for electron microscopic examination, we used a similar method. This procedure was as follows:

Method I. Ten ml of milk were centrifuged at 866 g (2,000 rpm) for 15 minutes to remove most of the fat. To the extracted milk 5 ml of phosphate buffer pH 7.5 was added containing 10 mg of chymotrypsin. This was mixed, incubated for 2 hours at 37°C and allowed to stand at 4°C overnight. The supernatant fluid was decanted from any precipitated protein and extracted with 15 ml of hexane to remove any remaining fat. One and a half ml of this solution was then centrifuged at 50,000 g (32,000 rpm) for 15 minutes. The resulting supernatant was drawn off by means of a curved needle and discarded. The precipitate was resuspended in 0.75 ml of distilled water, using a high speed stirring motor; the resulting suspension was again centrifuged at 50,000 g (32,000 rpm) for 15 minutes. The supernatant was discarded and the sediment was resuspended in 0.75 ml of distilled water, and centrifuged at 10,000 g (15,000 rpm) for 15 minutes, followed by 50,000 g (32,000 rpm)

for 2 minutes. The supernatant was now removed, placed in a clean centrifuge tube and diluted to 0.75 ml with distilled water. A holder containing an electro-mesh screen was then carefully introduced into the tube and a final centrifugation at 50,000 g (32,000 rpm) for 10 minutes deposited the material, to be examined, directly on the film supported by the electro-mesh screen. The screens were now removed, air dried, and after a preliminary direct electron microscopic examination, they were shadowed in a vacuum evaporator, at an angle of 10 to 15° at 10 cm distance, using 25 mg of chromium. In view of the fact that the results obtained with this method of segregation were not entirely satisfactory, much of the undifferentiated material remaining on the electron microscopic screen, another method was tried, apparently with more success. This second method was as follows:

Method II. Ten ml of milk were mixed with 30 ml of hexane in a separatory funnel at room temperature and allowed to settle for 15 minutes. After this period of time the bottom part of the mixture was removed from the funnel and centrifuged at 866 g (2,000 rpm) for 15 minutes. Five ml of the fat-free milk was now homogenized in a high speed tissue homogenizer with 35 ml of pure monochlorobenzene (specific gravity 1.12) for 5 minutes. The resulting emulsion was then centrifuged at 866 g (2,000 rpm) for 15 minutes. Three portions were then formed in the centrifuge tube, an aqueous supernatant layer, a middle layer of monochlorobenzene and a precipitate at the bottom. The middle layer was removed by means of a curved needle, by aspiration, and placed into a clean centrifuge tube. This was centrifuged at 10,000 g (15,000 rpm) for 15 minutes. The supernatant was removed, and monochlorobenzene added to make a total volume of 0.75 ml. This was again centrifuged at 17,500 g (20,000 rpm) for 10 minutes, the supernatant withdrawn, placed in a clean centrifuge tube containing the holder and electro-mesh screen, and once more monochlorobenzene was added to make a total volume of 0.75 ml. A final centrifugation was made at 50,000 g (32,000 rpm) for 3 minutes. The screen was now re-

TABLE I. Results of Electron Microscopic Examination of Milk Samples Collected from Healthy, Nursing Women, Having a Family History of Breast Cancer. (Group A).

Donor's record No.	Donor's family history	Results of electron microscopic examination of the milk samples*
1-A	Mother's 2 sisters had breast cancer	Particles present in fairly large numbers in clusters (+).†
2-A	Mother had breast cancer	Particles present in fairly large numbers in pairs and clusters (+).
3-A	Sister had breast cancer	Particles present in large numbers in clusters (++).
4-A	Mother and sister had breast cancer	Particles present in fairly large numbers (+).
5-A	Sister had breast cancer	Particles present in large numbers in pairs and clusters (++).
6-A	Mother had breast cancer	Particles present in large numbers in clusters (++).
7-A	Grandmother and 2 maternal aunts had breast cancer	Particles present in large numbers in pairs and clusters (++).
8-A	Mother had breast cancer	Particles present in large numbers in pairs and clusters (++).
9-A	Mother had breast cancer	Particles present in fairly large numbers (+).
10-A	Mother had breast cancer	Particles present in fairly large numbers (+).

\* Five of these samples were segregated by method I, and 5 by method II.

† Explanation of symbols: + particles present in fairly large numbers; ++ particles present in large numbers.

moved, air dried, and, after a preliminary direct electron microscopic examination, was shadowed in vacuum, at an angle of 10 to 15° from 10 cm distance with 25 mg of chromium. This second method of segregation proved to be more satisfactory than the first one, the background being clearer and containing less of undifferentiated debris. Of the 10 milk samples of group A, 5 were segregated by method I, and 5 by method II. Of the 32 control milk samples of group B, 9 were segregated by method I, and 23 by method II. Altogether, a total of 396 electron microphotographs was taken from all of the 42 human milk samples examined.

**Experimental.** *Results of electron microscopic examination of human milk samples.* Electron microscopic examination of 10 human milk samples collected from women having a family record of breast cancer (Group A), revealed in all 10 samples (Table I) the presence of small spherical particles (Fig. 1-5). These particles had a high density<sup>†</sup> to the electron beam; they appeared to have a regularly

round shape, and a smooth surface; their diameter varied from 20 to 200 m $\mu$ . These particles were found in at least some of the electron microscopic fields in pairs (Fig. 3), or clusters (Fig. 2, 4, 5). In 5 of the 10 samples examined, the particles were quite numerous. Essentially, they appeared similar to those found with the aid of an electron microscope in mouse milk<sup>(5,6)</sup> known to contain the mouse mammary carcinoma agent (Fig. 6).

The electron microscopic examination of 32 human milk samples that had been collected from women having a family record apparently free from malignant tumors for 2 preceding generations (Group B), revealed (Table II) in 11 of the 32 samples examined, the presence of multiple spherical particles, some of them in pairs, or clusters; in 5 of these 11 samples, the particles were in large numbers. Of the remaining 21 control samples of this group, the electron microscopic examination revealed in 17 samples the presence of only very few, mostly isolated, spherical particles (Fig. 7), occurring singly in some of the electron microscopic fields among masses of undifferentiated debris. In size and form, these particles were not different from those previously described,

<sup>†</sup> The density of the particles to the electron beam was determined by a direct electron microscopic examination of the film, prior to shadowing.



FIG. 1.

Electron-micrograph of a milk sample from a healthy, nursing woman, whose sister had cancer of breast (Rec. 5-A, Table I). Numerous spherical particles present frequently arranged in pairs or clusters. Chromium shadowed.  $\times 10,000$ .



FIG. 2.

Another electron-micrograph of same milk sample as that in Fig. 1 (collected from a woman whose sister had breast cancer). Multiple spherical particles in clusters. Chromium shadowed.  $\times 10,000$ .

except that they occurred only here and there, in most instances singly, and that at least some of them appeared to have a shallow notch on the top. Finally, 4 samples of milk of this control group appeared to be free from spherical particles, but contained some undifferentiated debris.

**Discussion.** Experiments reported in this study suggest that milk samples collected from

young, healthy, nursing women, whose sisters, mothers or grandmothers had breast cancer, but frequently also from those having a family record apparently free from tumors for 2 preceding generations, may contain spherical particles which can be visualized under an electron microscope. These particles have a diameter varying from 20 to 200 m $\mu$ . They have a smooth surface, a high density<sup>†</sup> to the electron beam, and occur, in some instances



FIG. 3.

Electron-micrograph of a milk sample from a healthy, nursing woman, whose grandmother and 2 maternal aunts had breast cancer (Rec. 7-A, Table I). Spherical particles in pairs and clusters. Chromium shadowed.  $\times 10,000$ .



FIG. 4.

Electron-micrograph of a milk sample from a healthy, nursing woman, whose mother had breast cancer (Rec. 8-A, Table I). Numerous spherical particles in clusters. Chromium shadowed.  $\times 10,000$ .



FIG. 5.

Electron-micrograph of a milk sample from a healthy, nursing woman, whose sister had breast cancer (Rec. 3-A, Table I). Numerous spherical particles in clusters. Chromium shadowed.  $\times 10,000$ .

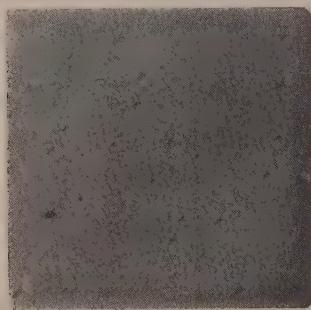


FIG. 6.

Electron-micrograph of a *mouse* milk sample containing the mammary carcinoma agent. This sample was obtained in the following manner: Five litters consisting of a total of 24 suckling infants, 4 to 6 days old, born to mice of the C3H line known to carry the mammary carcinoma agent, were sacrificed; the cheese-like contents were removed aseptically from the stomachs of these infants, and mixed in a mortar with 10 ml of physiological saline solution; the resulting milk-like suspension was then treated exactly like a human milk sample by our second method of segregation. Chromium shadowed.  $\times 11,200$ . Numerous spherical particles, having a diameter varying from 20 to 200  $\mu$ , very similar to those found in human milk (compare with Fig. 1 and 2).

at least, in pairs, or clusters. In their morphology, size, and group arrangement, these particles appear to be similar to those observed in milk collected from mice known to carry the mammary carcinoma agent (5,6).

The nature of these particles remains obscure. Theoretically, it would be possible to assume that they are (a) normal components of human milk, or that they (b) represent some accidental virus infection unrelated to cancer, or that they (c) actually represent a human cancer agent, essentially similar to the mouse mammary carcinoma virus. It is also possible to assume that (d) some of the particles, such as those present in large numbers, in pairs and clusters, may represent the tumor agent, while other particles, similar in certain respects, but slightly different in others (such as those having a notched top, isolated appearance, etc.) may be either normal components of human milk, similar to those described in normal mouse cells (7), or may have some other origin; small spherical particles have been found with the aid of the electron microscope in various biological samples, recently also in some samples of cow milk (10,11) as well as in samples of mouse milk apparently free from the mammary carcinoma agent (6,11).

Should all these particles be normal com-

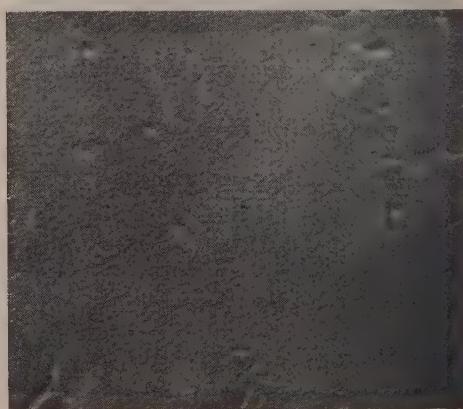


FIG. 7.

Electron-micrograph of a control milk sample, collected from a healthy, nursing woman, who had a family record apparently free from cancer. Only occasional, few particles present in some of the electron-microscopic fields. Chromium shadowed.  $\times 10,000$ .

10. Nitschmann, H., *Helvetica Chimica Acta*, 1949, v32, 1258.

11. Gross, L., Gessler, A. E., and McCarty, K. S., Experiments to be published.

TABLE II. Results of Electron Microscopic Examination of Milk Samples Collected from Healthy, Nursing Women Having a Family History Presumably Free from Cancer. (Group B).

No. of milk samples examined (each from a different donor)	32
Results of electron microscopic examination	
No. of samples* with numerous particles in pairs and clusters (++)	5
No. of samples† with less numerous particles, in pairs or clusters (+)	6
No. of samples‡ with only occasional single particles	17
No. of samples§ with apparently no particles present	4

\* 4 of these samples segregated by method I, and 1 by method II.

† One of these samples segregated by method I, and 5 by method II.

‡ 4 of these samples segregated by method I, and 13 by method II.

§ All 4 samples segregated by method II.

ponents of human milk, one would rather expect them to be present in fairly equal numbers, and group formations, in all of the samples examined. This was not the case, however. These particles were present in fairly large numbers, and quite often in characteristic pairs, or cluster formations, in all of the 10 milk samples collected from women having a family record of breast cancer. Of the 32 control samples, however, collected from women having a presumably negative family history for cancer, only 11 contained the characteristic groups of spherical particles in either fairly large, or large numbers; the remaining 21 control samples contained in most instances only single, isolated particles in some of the electron microscopic fields (17 samples), or apparently none at all (4 samples). It also remains open to question whether all of the donors of these 32 control samples were actually descendants of families free from cancer; tumors, including cancer of the breast, might well have developed in some of the ancestors of these women-donors, even though this information might not have been available to the interviewing physicians.

The presence of spherical particles in human milk samples is interesting in view of similar findings recorded in mice. Spherical particles, similar to those reported in this study, were found in samples of milk collected from mice known to carry the mammary carcinoma agent(5,6). When sediment containing

such particles was injected into susceptible mice, mammary carcinomas developed in these animals as a result of inoculation(5,6). When normal mouse tissue(6,8), or normal mouse milk(5,6,11) from mice of low-tumor lines, were examined with the electron microscope, spherical particles were also found, but less frequently(5,6,8,11).

Similar spherical particles were also observed with the electron microscope in human cancer(12,13), but less frequently in normal, healthy, human tissues(13).

The wide range of the diameter size of the particles observed in human milk, as well as of those found in mouse milk and mouse tumors (20 to 200 m $\mu$ ) may, at first, appear to speak against the assumption that these particles represent a tumor agent. And yet it should be kept in mind that electron microscopic examination of several viruses, such as that of mumps(14), or psittacosis(15); also revealed wide variations in their respective sizes.

Interesting as these considerations may be, it should be emphasized, nevertheless, that no definite conclusions can be reached at the present time concerning the nature of the particles observed in human milk. They may, or may not be associated with the hypothetical cancer agent. The only conclusion possible at the present time is that the electron microscopic examination of 10 samples of human milk collected from women having a family history of breast cancer revealed the presence of small spherical particles more frequently than that of 32 samples obtained from women with a family record apparently free from cancer.

**Summary.** 1. Ten samples of human milk collected from young, healthy, nursing women, whose sisters, mothers, or grandmothers had

12. Gessler, A. E., and Grey, C. E., *Exp. Med. and Surg.*, 1947, v5, 307.

13. Gessler, A. E., McCarty, K. S., Parkinson, M. C. and Bardet, J. M., *Exp. Med. and Surg.*, 1949, v7, 237.

14. Weil, M. L., Beard, D., Sharp, D. G., and Beard, J. W., *Proc. Soc. EXP. BIOL. AND MED.*, 1948, v68, 309.

15. Heinmets, F., and Golub, O. J., *J. Bacteriol.*, 1948, v56, 509.

breast cancer, were examined with the aid of an electron microscope. Spherical particles, of a smooth surface and a high density<sup>†</sup> to the electron beam, varying in diameter from 20 to 200 m $\mu$ , in some instances grouped in pairs, or clusters, were found in all samples; they were particularly numerous, however, in 5 of the 10 samples examined. These spherical particles appeared to be similar to those previously observed in mouse milk known to contain the mouse mammary carcinoma agent.

2. Thirty-two control human milk samples, collected from young, healthy, nursing women having a family record apparently free from any malignant tumors for 2 preceding generations, were also examined with the aid of an electron microscope. Eleven of them were found to contain spherical particles essentially similar to those described above. Of the remaining 21 control milk samples, 17 were found to contain only occasional, isolated, single particles in some of the electron micro-

scopic fields; the other 4 samples appeared to be free from spherical particles, but contained some unidentified debris.

3. No definite conclusions can be reached at this time as to the nature of the spherical particles revealed with the aid of the electron microscope in human milk.

Twenty-nine human milk samples were processed, and examined with an RCA electron microscope type EMB at the Research Laboratories of the Interchemical Corporation, New York; Mr. M. Parkinson, Mr. J. J. Kelsch, Mr. R. Bainbridge, and Miss Joan Bardet rendered valuable technical assistance in this part of our work. Thirteen human milk samples and several mouse milk samples were examined with an RCA electron microscope type EMU-2B, at the Research Laboratories of the Veterans Administration Hospital, Bronx, New York.

Most of the human milk samples were obtained from the New York Lying-In Hospital. Several samples were obtained through the courtesy of Dr. Milton J. Goodfriend, New York.

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### Relative Susceptibility of Various Stocks of Mice to Experimental Disseminated Encephalomyelitis.\* (18169)

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The present paper extends recent studies on experimental disseminated encephalomyelitis induced in white mice by means of injection of homologous brain plus Freund-type adjuvant(1) and offers further results of tests on the variation of susceptibility of certain stocks of mice. Certain aspects of the experimental affection will also be shown. A wide difference has been found(1) in the suscepti-

bility of the W-Swiss(2) and the Rockefeller Institute(3) strains of mice, the former being susceptible, the latter resistant. Thus in an experiment in which the same method and materials were employed, 19 of 20 injected Swiss mice, while only 1 of 15 Rockefeller Institute mice developed encephalomyelitis(1).

The tests now to be described included a study of additional stocks of mice: (a) W-Swiss mice, H-line, of the same strain as was hitherto employed and found to be reactive to inoculation of homologous brain with development of encephalomyelitis. (b) Another stock was added, W-Swiss B-line, obtained from a source outside of the laboratories but

\* The writers express their gratitude to Major L. C. Murphy, V.C., U. S. Army, and to Joan Fitzgerald for their assistance, and to Dr. Howard A. Schneider of the Rockefeller Institute for supplies of mice.

† Public Health Research Fellow of the National Institute of Mental Health.

1. Olitsky, P. K., and Yager, R. H., *J. Exp. Med.*, 1949, v90, 65.

2. Webster, L. T., *J. Exp. Med.*, 1939, v70, 87.

3. Webster, L. T., *J. Exp. Med.*, 1933, v57, 793; 1937, v65, 261.

propagated for more than ten years as an inbred strain deriving originally from W-Swiss H-line parents. This B-line stock was developed by selection so as to obtain certain uniform litters, and was nurtured under conditions different from those of the H-line. At present it may be regarded as a separate stock. (c) A third stock comprised a line developed from piebald house mice, a true breeding variety called white-faced(4), maintained as inbred for several years by Dr. H. Schneider.

*Method and materials.* Any of the following preparations of mouse brain-adjuvant mixtures was used without much difference in the outcome: (a) fresh mouse brain obtained from normal-appearing Rockefeller Institute or W-Swiss strain mice, 10 g; autoclaved tubercle bacilli H37Rv type(1), 20 mg; liquid petro-latum (Soconal), 50 cc; and 0.85% saline solution, 50 cc. (b) This preparation was similar to the aforementioned one except that the amount of tubercle bacilli was increased to 250 mg. (c) This preparation was similar to (b) except that light mineral oil and Falba 0.5 g was substituted for Soconal. The injections of 0.3 cc were given, as a rule, subcutaneously and rarely intramuscularly at 7-day intervals until either a total of 10 injections had been given or characteristic signs appeared earlier. Mice 1 to 2 months old were used; they were observed 2 to 3 months after the first introduction of the brain-adjuvant mixture. The picture presented by the reactors has already been described(1). On admission to the laboratory all animals were kept under the same environmental conditions and given the same diet of fox chow, bread and milk, and water.

*Experimental.* The tests which were made in addition to those already reported(1) are summarized and tabulated (Table I). It will be noted that among 65 mice of the W-Swiss H-line, 52 developed disseminated encephalomyelitis after multiple injections of homologous brain-adjuvant mixtures, the average number of inoculations being 4 in one experiment and 6 in two others. As already described this stock of mice has been shown

to be susceptible: of 50 injected mice 46, or 92%, exhibited the experimental affection(1). In comparison with that reactive stock, the W-Swiss B-line showed a reverse effect, namely, a relative resistance to the development of the experimental disorder, *i.e.* of 45 injected mice only 9 were found to be reactors. Finally, of the 12 injected white-faced mice only one came down with the neurological syndrome. Thus the latter animals join the Rockefeller Institute strain(1) in the class of those that are refractory. It appears that there is a constitutional or endogenous factor in certain stocks which marks the animals thereof as susceptible or relatively resistant to the effects of the "antigen" that produces disseminated encephalomyelitis in mice.

It is also to be observed from the table that once an animal reacted to the injections, the time taken before the first sign was manifested, or the number of inoculations needed to produce the disorder, did not differ in animals that were susceptible or were relatively resistant. The only exception was the single white-faced mouse that reacted. Here the time taken for the first signs to become visible, and the number of injections given were both greater.

It should be stressed, moreover, that of the 62 animals which reacted 12, or 19%, exhibited a variation in degree and number of lesions in the nervous system characteristic of disseminated encephalomyelitis, even though none of the 12 revealed any objective sign of illness.

*Discussion.* Multiple sclerosis and other demyelinating diseases are brought into this discussion because investigators of the problem of experimental disseminated encephalomyelitis have drawn attention to the similarity—not, however, the identity—of the symptoms and pathological picture induced in lower animals to those found in the human affections. Moreover, in multiple sclerosis the prevailing theory of its origin includes two factors: (a) constitutional or familial or endogenous susceptibility which by itself is ineffective to bring about the disease and (b) an exogenous agent, or perhaps agents, which can induce the disorder especially when the

## MURINE DISSEMINATED ENCEPHALOMYELITIS

TABLE I. Showing Variation in Susceptibility of Different Stocks of Mice.

	No. of mice used	Stocks of mice						Mice with no signs only CNS lesions
		W-Swiss H-line		W-Swiss B-line		White-faced		
		Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	
Exp. I*	52	16	9	3	12	1	11	H-line, 3; B-line, 1
Avg days to 1st sign		42		41		64		
Avg No. inj.		6		5		9		
Exp. II	25	21	4					H-line, 4
Avg days to 1st signs		52						
Avg No. inj.		6						
Exp. III	30	15	0	0		15		
Avg days to 1st signs		29						
Avg No. inj.		4						
Exp. IV	15			6	9			B-line, 4
Avg days to 1st signs				32				
Avg No. inj.				5				
Totals	122	52	13	9	36	1	11	12
Approx. %		80	20	20	80	8	92	19 (of 62)

\* In Exp. I, the W-Swiss H-line mice were maintained by others than those who supplied animals for Exp. II and III.

former, or endogenous factor, has first prepared the way(5). Any constitutional element influencing the production of encephalomyelitis has not been brought forward for the experimental animals hitherto studied by others. It was shown here, however, to exist in the mouse which served as a delicate indicator of the importance of the stock from which the animal is taken for the production of the neurological syndrome. The W-Swiss H-line was found to be susceptible and a separate variety, the W-Swiss B-line, relatively resistant, even though the latter was derived from the same original W-Swiss strain. Also resistant was the Rockefeller Institute strain, as previously shown(1) and the white-faced mice, now demonstrated.

One should not look here for a direct relation to the problem of human demyelinating diseases since it is yet to be proved that experimental disseminated encephalomyelitis induced in any species of animal is identical with them. It is, however, of interest that in the experimental encephalomyelitis which does bear certain resemblances to the human maladies an endogenous factor, as found in animals of certain stocks, should be correlated with susceptibility or resistance.

5. Merritt, H. H., Wortis, S. B., and Wolftman, H. W., Foreword in Multiple Sclerosis and the Demyelinating Diseases, *Res. Publ. Assn. Nerv. Ment. Dis.*, 1950, v28, 1-675.

Another point of interest is the number of injected mice that showed no outward sign of illness yet at autopsy varied in the degree and extent of lesions in the central nervous system, chiefly vascular and perivascular, of a nature already described(1). These were noted most often in the white substance and granular layer of the cerebellum; in periventricular areas, especially the white matter about the IV ventricle; in the anterior thalamic regions, and in the dorsal spinal cerebellar tracts and fasciculi. It is clear, first, that considerable damage can exist in the nervous system of the mouse without observable, objective signs of illness. Moreover, since these histopathological changes resulted specifically from the injections of the brain tissue-adjuvant mixtures, it becomes necessary to examine animals apparently symptom-free for the presence of such lesions before they can be declared non-reactive. Finally, if what occurs in the mouse is a reflection of what may exist in other experimental animals in this type of work, *viz.*, monkeys, rabbits and guinea pigs, a clear indication arises to examine the central nervous system of all such as exhibit no symptoms before they can be considered negative.

**Summary.** 1. The stock from which the host is taken is apparently important for the outcome of attempts to induce in mice experimental disseminated encephalomyelitis,

for certain stocks of albino mice are susceptible and others, even though of related lines, are relatively resistant. 2. In 19% of the mice which reacted to homologous brain tissue-Freund type adjuvant mixtures, lesions characteristic of disseminated encephalomyelitis can be found in the central nervous system in the absence of any demonstrable objective symptoms of illness. 3. A diagnosis of the experimental encephalomyelitis should there-

fore be based on the results of histological examination of the nervous system as well as on typical symptomatology. The question is discussed whether reactors might not have been overlooked among animals of other species hitherto employed, when the basis for the diagnosis of the encephalomyelitis was only the presence of outward signs of illness.

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### Role of the Erythrocyte in Inhibition by Allantoic Fluid of Mumps Virus Hemagglutination. (18170)

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It is known that virus hemagglutination is the result of a reaction between the virus and the red blood cell, and that this reaction may be specifically inhibited by sera containing antibodies for the virus. A variety of non-specific substances may also interfere with this reaction(1). A substance of this sort present in allantoic fluid has been studied most intensively for its effect upon the agglutination of red blood cells by influenza viruses(2,3). From these investigations, it appears that normal allantoic fluid (NAF) contains an inhibitor which reacts with and is inactivated by these viruses. A filtrate of *Cl. welchii* and an enzyme from *Vibrio cholera* similarly inactivate this inhibitor. It has been postulated that a coenzyme exists for the hemagglutinating virus-inhibitor system. However, it has not been clear whether this hypothetical substance comes from the virus or from the red blood cell(2).

In the course of comparative studies on agglutination of chicken and human erythrocytes by a strain of mumps virus (Enders), observations were made which seem to furnish information regarding this question. These observations are the subject of the

present communication.

*Materials and methods. Viruses* (a) *Mumps*. The Enders strain was used. For passage 0.1 ml of a  $10^{-1}$  dilution of virus in 10% rabbit serum-saline was inoculated into the allantoic sac of 7-day-old chick embryos. These embryos were incubated further at 37°C for 4 days, chilled and the allantoic fluid collected. (b) *Influenza*. The PR-8 and Lee strains were used. For passage 0.1 ml of a  $10^{-3}$  dilution of virus in 10% rabbit serum-saline was inoculated into the allantoic sac of 10- to 11-day-old chick embryos. The allantoic fluid was collected after 2 days at 37°C and a period of chilling.

*Normal allantoic fluid* (NAF) was obtained from chilled 11-day-old embryos. All the fluids were stored after collection in lusteroid tubes at approximately -72°C.

*Chicken red blood cells* were obtained by bleeding chickens from the wing vein and allowing the blood to flow into an excess of 2% sodium citrate or sodium oxalate.

*Human group O cells* were obtained from the Hospital Blood Bank where they had been collected in ACD (acid citrate dextrose) solution. The cells were washed in normal saline 3 times and used as 1% or 25% suspensions as indicated. In some experiments other anticoagulants were used without modifying the results. Cells were not used if

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2. Svedmyr, A., *Brit. J. Exp. Path.*, 1948, v29, 309.

3. Hardy, P. H., Jr., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1948, v88, 463.

they were more than 3 days old.

*Hemagglutinin titrations.* Serial two-fold dilutions of virus containing allantoic fluid were made in saline in a volume of 0.4 ml. To each dilution, 0.4 ml of a 1% cell suspension was added. The tubes containing these mixtures were shaken and examined after being permitted to settle at room temperature for one hour. The pattern at the bottom of each tube was noted and graded from a 4+ shield-like pattern to 0, which represented a button of freely movable cells. The highest dilution of virus giving definitely positive agglutination (+1), was considered as the end point, representing 1 hemagglutinating unit of virus. *Inhibitor titrations.* Serial two-fold dilutions of allantoic fluid were made in saline in a volume of 0.2 ml. To each dilution, a specified number of hemagglutinating units of virus were added in a volume of 0.2 ml. Finally 0.4 ml of a 1% cell suspension was added. The tubes were shaken and the precipitated cell pattern observed after 1 hour at room temperature. These were graded as in the hemagglutinin titrations.

*Absorptions of virus.* Equal volumes of virus containing allantoic fluid and 25% cell suspensions (usually 0.5 ml of each) were mixed in a series of tubes. These were shaken frequently while at 37°C and after a specified period, quickly centrifuged, so that the supernatant fluid could be readily removed. The fluid was then titrated for residual hemagglutinin content using a 1% suspension of chicken cells. The period of contact between 25% cell suspension and allantoic fluid usually varied between 2 and 62 minutes, including the period of centrifugation.

*Experimental. Agglutination of chicken and human erythrocytes by mumps virus.* The Enders strain of mumps virus agglutinates both chicken and human red blood cells. Other strains (e.g. Habel) appear to agglutinate only chicken cells(4). It is known that allantoic fluids from different eggs contain varying amounts of hemagglutination inhibitor(3,5,6). However, from our present

TABLE I. Titration of Allantoic Fluid Infected with Mumps Virus Using Chicken and Human Red Blood Cells.

Fluid	Cells	Final dilution of fluid							
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
151-1	C	4	4	4	4	4	4	4	0
	H	3	4	4	4	4	2	0	0
	C	4	4	4	4	4	4	4	0
	H	0	0	0	0	0	0	0	0
	C	4	4	4	4	4	4	4	0
	H	0	0	+	1	1	0	0	0
	C	4	4	4	4	4	4	4	0
	H	3	3	4	4	4	4	2	0
	C	4	4	4	4	4	3	2	0
	H	0	2	3	4	4	2	0	0

C = Chicken.

H = Human.

studies it seems clear that the inhibitor for mumps (Enders) hemagglutination in any particular sample of infected allantoic fluid is more apparent when human rather than chicken cells are used. This is illustrated in Table I which gives the results of a representative experiment in which fluids from embryos with titers with chicken cells which are comparable are also examined with human cells. Among 11 serial passages of mumps virus in which undiluted fluid from 78 individual embryos were tested, it was found that only 48% of those which agglutinated chicken cells also agglutinated human red blood cells. Yet many of the remaining 52% did agglutinate human cells almost as well as they did chicken erythrocytes when they were diluted beyond the effective range of the inhibitor (Table I).

*Absorption of mumps virus by chicken and human erythrocytes.* Further evidence of the greater activity of this inhibitor in the presence of human cells than in the presence of chicken cells was obtained from absorption studies. Fig. 1 presents results of representative experiments done with fluids which did not appear to have excess inhibitor, since when used undiluted they agglutinated human cells. Nevertheless, clear evidence of absorption of virus was obtained only with

4. Florman, A. L., Unpublished experiments.

5. Beveridge, W. B., and Lind, P. E., *Austral. J. Exp. Biol. and Med. Sci.*, 1946, v24, 127.

6. Lind, P. E., *Austral. J. Exp. Biol. and Med. Sci.*, 1948, v26, 93.

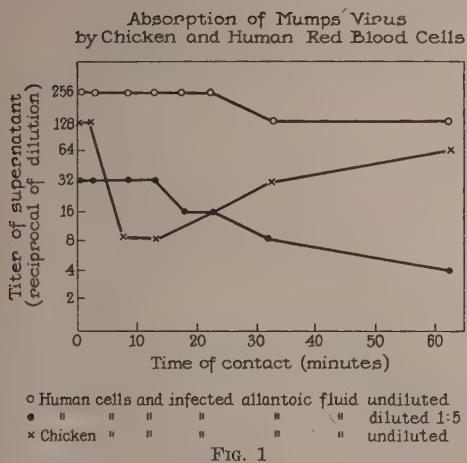


FIG. 1

chicken cells. When human cells were used there was no apparent absorption, unless the allantoic fluid was first diluted. Even then the absorption was definitely slower than with chicken cells. In other experiments, using similar suspensions of chicken and human cells with influenza virus, it was easy to demonstrate absorption of virus from undiluted infected allantoic fluids by both species of cells.

*Effect of NAF and mumps infected allantoic fluid on small quantities of mumps virus in presence of human cells.* Normal allantoic fluids diluted as high as 1:64 and 1:128 were capable of preventing agglutination of human cells by two and four units of mumps virus. This effect was less apparent as the amount of virus was increased. However, it was significant that doubling the amount of virus did not give a proportionate decrease in inhibitor activity. Allantoic fluids from several mumps infected embryos acted in a similar fashion though quantitatively much less effectively. The inhibitor effect was not noted beyond a dilution of 1:8 and 1:16. It seemed that a considerable amount of the inhibitor had been inactivated by the virus already present in this infected fluid.

*Comparative inhibiting effect of NAF on agglutination by mumps virus of chicken and human cells.* In order to determine whether the apparently greater inhibiting effect of allantoic fluid on mumps virus agglutination

of human red blood cells is not merely a reflection of lesser sensitivity of human cells for the virus, the experiment summarized in Table II was carried out. A single pool of mumps virus was diluted as indicated. Different amounts of NAF were added to aliquots of each dilution. To one set of each pair there was added a suspension of chicken cells and to the other set a similar suspension of human cells. The linear reduction of titer when chicken cells are employed stands in striking contrast to the precipitous reduction when human cells are used. A comparison of the effect in the presence of 2 and 4 units of virus in each set is especially noteworthy. It is consequently clear that the inhibitor is more active in the presence of human cells, and that the relative sensitivity of these cells for the virus did not determine the observed results.

*Attempts to modify the mumps virus-inhibitor reaction in presence of human cells.* The use of infected allantoic fluids of varying ages (2 to 105 days), failed to modify the nature of the mumps virus-human red blood cell absorption curve. Variations in pH from 6.8 to 8.4 did not change this reaction, nor did the use of undiluted allantoic fluid which had been previously exposed for 1 hour to a 25% suspension of human cells.

*Comparison of effect of NAF on agglutination by mumps and influenza viruses of chicken and human erythrocytes.* In Table III there are presented the results obtained when small quantities (2 agglutinating units) of mumps and influenza viruses were mixed

TABLE II. Inhibitory Effect of NAF on Agglutination by Mumps Virus of Chicken and Human Cells.

Cells	NAF	Final dilution of virus						
		1:4	1:8	1:16	1:32	1:64	1:128	1:256
C	0	4	4	4	4	4	0	0
C	1:4	4	4	3	1	0	0	0
C	1:8	4	4	4	4	1	0	0
C	1:16	4	4	4	4	2	0	0
H	0	3	4	4	3	0	0	0
H	1:4	0	0	0	0	0	0	0
H	1:8	0	0	0	0	0	0	0
H	1:16	0	0	0	0	0	0	0

*Italic figures = 2 and 4 units of virus present.*

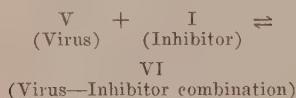
TABLE III. Effect of NAF on Agglutination of Chicken and Human Red Blood Cells by Mumps and Influenza Viruses.

Virus*	Cells	Final dilution of NAF							
		1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Mumps	C	0	0	0	±	2	3	3	2
	H	0	0	0	0	±	1	2	3
Lee	C	0	±	3	4	4	4	4	4
	H	4	4	4	4	4	4	4	4
PR-8	C	0	0	1	2	3	3	3	3
	H	0	4	4	4	4	4	4	4
Saline	C	0	—	—	—	—	—	—	0
Controls	H	0	—	—	—	—	—	—	0

\* 2 hemagglutinating units present in each instance.

with varying amounts of NAF and tested with chicken and human erythrocyte suspensions. Although the results with mumps virus are most striking, they are not essentially different from those obtained with PR-8 and Lee viruses. However, in contrast to the findings with mumps virus, with the influenza viruses the effect is more obvious with chicken cells than with human cells.

*Discussion.* The results of these experiments suggest that with mumps virus the equation introduced by Hardy and Horsfall (3) to explain the virus-inhibitor reaction:



is influenced in the presence of human cells to move in the direction of combined virus (VI). Consequently, agglutination which depends upon the presence of free virus may not be apparent until the fluid is diluted beyond the zone of effective activity of the inhibitor. Since this same fluid before dilution may show clear agglutination of chicken cells, it would seem that in the presence of chicken cells the equation is influenced in the reverse direction (in the direction of free virus). With PR-8 and Lee viruses the same type of activity can be presumed to take place, with the exception that in those systems the chicken cells furnish something which influences the equation in the direction of combined virus.

*Summary.* It is shown that the inhibitor present in allantoic fluid for hemagglutination by and absorption of mumps virus is more active when human erythrocytes rather than when chicken red blood cells are used. It appears that the species of erythrocyte present influences the reaction between mumps virus and inhibitor in the direction of more or less combined (non-hemagglutinating) virus. A similar influence, though to a less striking degree, is also shown for the red blood cell in the influenza virus-inhibitor reaction.

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### Development of Fatty Livers in Fasted Male Mice Bearing a Transplantable Lymphosarcoma.\* (18171)

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A variety of experimental conditions in-

\* This investigation was aided by a grant (to Abraham White) from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

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cluding dietary alterations, endocrine treatment, and the administration of toxic substances, are known to result in the development of fatty livers in laboratory animals (1). During the course of an investigation

1. McHenry, E. W., and Patterson, J. M., *Physiol. Rev.*, 1944, v24, 128.

of the influence of a rapidly growing transplantable lymphosarcoma on the metabolism of the host, accumulation of lipid has been noted in the livers of tumor-bearing male mice fasted for 48 hours. By contrast, fatty livers did not appear in similar tumor-bearing mice fed the normal laboratory diet of Rockland chow, or in normal male mice fasted for 48 hours under the same conditions.

**Methods.** Male mice of the CBA strain (Strong) weighing 20 to 25 g and 8 to 9 weeks of age were used. Sex, age and strain specification is of the utmost importance, since it is well known that such variables influence the susceptibility of animals to the development of fatty livers on fasting alone. Three-month-old albino male mice under specified conditions have been reported(2) to develop fatty livers after a 24- to 48-hour fast, although the relation of maximum liver fat to day of fasting could be modified by changes in the environmental temperature. Similarly it has been noted in connection with the observations to be reported that older CBA male mice at about 16 weeks of age also develop fatty livers as the result of a 48-hour fast alone. For these reasons, it must be emphasized that at the termination of a 48-hour fast under the conditions of the present observations, the livers of normal CBA male mice 7 to 10 weeks of age show only a slightly increased percentage of lipid, and (because of considerable loss in liver weight), a prominent reduction of total lipid, expressed both in absolute quantity and relative to body weight(3).

Mice prepared for fasting experiments were inoculated subcutaneously with a small fragment of a transplantable lymphosarcoma(4). At varying intervals thereafter, groups of 5 to 7 animals were subjected to a 48-hour fast with free access to water, while control groups received the laboratory diet. At the end of the fasting period each mouse in both

groups was autopsied and aliquots of liver tissue analyzed for water content by drying at 110°C, and for nitrogen concentration by the micro-Kjeldahl procedure(5). From these data, liver lipid concentration was calculated by difference, a method whose validity has been established by comparison with direct chemical analysis for total lipid(3). Mice of similar age<sup>†</sup> were bilaterally adrenalectomized 6 to 9 days following tumor inoculation and were subjected to a 48-hour fast beginning 24 to 48 hours following operation. All adrenalectomized animals were maintained continuously on 1% saline as drinking fluid. All experimental mice, either fasted or fed, were kept in individual metabolism cages for the duration of the 48-hour fasting period. The environmental temperature was not held constant but was generally between 20 and 25°C.

**Results.** The livers of fasted tumor-bearing mice in almost all groups were visibly fatty on gross inspection. Under these conditions, mice free of tumor did not develop fatty livers as the result of fasting alone, as has been previously demonstrated(3). Similarly, the presence of the tumor in fed mice, caged under identical conditions of temperature and opportunity for activity, never resulted in the development of fatty livers. The opposite trend, in fact, is indicated by the data shown in Table I, i.e., a tendency for the livers of tumor-bearing fed mice to contain both a slightly lower concentration and total content of lipid, as compared with fed mice free of tumor.

As is evident from the data presented, a 48-hour fast resulted in distinctly fatty livers at varying intervals after tumor implantation, and without quantitative correlation with tumor size. This phenomenon did not occur either very early (2 days) following tumor inoculation, or very late in the development of the tumor, at a time when the mice appeared moribund. It is notable, however, in the data of Experiment 4 that markedly fatty

2. Hodge, H. C., Machlachlan, P. L., Bloor, W. R., Welch, E., Kornberg, S. L., and Falkenheim, M., *PROC. SOC. EXP. BIOL. AND MED.*, 1948, v67, 137.

3. Szego, C. M., and White, A., *Endocrinology*, 1949, v44, 150.

4. Gardner, W. U., Dougherty, T. E., and Williams, W. L., *Canc. Res.*, 1944, v4, 73.

5. Sobel, A. E., Yuska, H. and Cohen, J., *J. Biol. Chem.*, 1937, v118, 443.

† The mice of Experiment XI were 9 to 10 weeks of age at the time of autopsy.

## FATTY LIVERS WITH MOUSE LYMPHOSARCOMA

TABLE I.\* Effect of a 48-hour Fast on Concentration of Liver Lipid in Fed and Fasted Unoperated and Adrenalectomized Lymphosarcoma-bearing Male Mice. Each experiment represents paired groups of mice autopsied at the same time, with the exception of Exp. V, X, and XI, in which an initial fed group was autopsied at the beginning of a 48-hour fasting period.

Exp.	Group	No. of mice	Tumor age (days)	Tumor wt (mg)	Body wt (g)	Liver wt (mg)	% liver lipid
Normal mice							
I	Fed	7	—	—	25.3 ± .6	1800 ± 76	10.4 ± .9
II	Fed	5	—	—	21.6 ± .3	1330 ± 42	10.3 ± .4
	Fasted	5	—	—	17.6 ± .7	960 ± 33	10.8 ± 1.3
Tumor-bearing mice							
III	Fed	5	2	<10	20.0 ± .9	1250 ± 33	10.0 ± .8
	Fasted	5	2	<10	15.8 ± .8	750 ± 47	10.0 ± 1.3
IV	Fed	5	6	<10	20.9 ± .9	1260 ± 25	9.6 ± 1.0
	Fasted	5	6	<10	15.9 ± .8	1090 ± 72	18.8 ± 1.5
V	Fed	5	4	<10	22.4 ± 1.2	1370 ± 70	9.4 ± .2
	Fed	4	6	279 ± 40	22.1 ± .7	1350 ± 66	10.6 ± .3
	Fasted	5	6	15 ± 10	16.8 ± .7	1270 ± 57	27.1 ± .9
VI	Fed	6	7	530 ± 28	24.5 ± .5	1650 ± 56	7.6 ± 1.0
	Fasted	5	7	80 ± 31	16.4 ± .3	1120 ± 39	16.0 ± .7
VII	Fed	6	9	177 ± 30	24.0 ± .8	1420 ± 56	7.3 ± .4
	Fasted	6	9	117 ± 35	19.0 ± .7	1180 ± 26	11.8 ± 1.6
VIII	Fed	6	11	1580 ± 233	26.2 ± .8	1570 ± 58	8.5 ± .7
	Fasted	6	11	428 ± 112	19.2 ± .7	1350 ± 75	13.9 ± .3
IX	Fed	3	15	3580	30.2	1630	9.0
	Fasted	2	15	2340	21.1	900	6.1
Adrenalectomized tumor-bearing mice							
X†	Fed	5	7	<10	20.5 ± .8	1240 ± 99	7.4 ± .9
	Fed	5	9	244 ± 108	21.2 ± .7	1170 ± 9	8.2 ± .3
	Fasted	7	9	150 ± 60	21.2 ± 1.0	1080 ± 32	15.1 ± .9
XI‡	Fed	5	11	527 ± 60	22.6 ± 1.0	1270 ± 107	8.9 ± 1.2
	Fed	4	13	1060 ± 221†	22.8 ± .9	1300 ± 74	8.9
	Fasted	6	13	590 ± 102	20.9 ± .5	1220 ± 67	16.9 ± 2.9

\* Values shown are means and stand. errors. Stand. errors were not calculated for groups of less than 4 mice.

† Represents group of 7 mice.

‡ Adrenalectomized on the 6th day following tumor implantation.

§ Adrenalectomized on the 9th day following tumor implantation.

|| Mean of 3 values.

livers were seen in fasted mice in the presence of tumors of minute size, not grossly palpable.

In 2 instances (see Table) experiments similar to those above were carried out with lymphosarcoma-bearing fasted mice which had been bilaterally adrenalectomized 1 or 2 days prior to the beginning of the fasting period. In such animals no inhibition of the development of fatty livers on fasting appeared to result from the absence of the adrenals.

**Discussion.** These observations, describing the appearance of fatty livers in fasted mice carrying a transplantable lymphosarcoma, throw no light on the mechanism of its production. Indeed, the specificity of this phenomenon for tumors of specific cellular type, or for stresses of more generalized character,

requires further investigation. It is conceivable that the presence of a rapidly growing tumor, like exposure to cold(6), represents an additional demand for calories beyond that required by a fasted animal, and that the accumulation of excessive liver lipid may result from the increased mobilization of depot fat necessary to meet this additional caloric demand. That the presence even of a minute tumor, seemingly too small to create a significant caloric demand by virtue of its own energy metabolism, is sufficient to determine the development of a fatty liver in the fasted host, suggests the alternate possibility of a more specific, perhaps humoral, influence on

fat metabolism. Furthermore, the persistence of lipid accumulation in the absence of the adrenals would seem to distinguish this type of fatty liver from those described in previous studies(6,7) which implicated the adrenal cortex in the mobilization of fat to the liver under circumstances of stress. In this connection, it may be of significance that one of the few experimental procedures which result in fatty livers in fasted male mice and is similarly effective even in the absence of the adrenals, is the administration of purified growth hormone(3).

7. MacKay, E. M., and Barnes, R. H., *Am. J. Physiol.*, 1937, v118, 525.

**Summary.** The development of fatty livers has been noted in 8-week-old fasted CBA male mice bearing a transplantable lymphosarcoma of varying age. Similar mice free of the tumor fail to develop fatty livers following a 48-hour fast under comparable conditions, nor does the growth of the tumor in fed mice result in fatty livers. Adrenalectomy has no inhibitory effect on the development of a fatty liver under these conditions.

Mrs. Rita Huang contributed valuable assistance with the analytical and operative procedures.

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### Urinary Excretion of Certain Amino Acids During ACTH and Cortisone Treatment of Rheumatoid Arthritis.\* (18172)

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In a previous publication from this laboratory(1) it was shown that the urinary excretion and plasma levels of free histidine were increased in patients with rheumatoid arthritis treated with ACTH (adrenocorticotropic hormone) and Cortisone (17-hydroxy-11-dehydro corticosterone). Urinary excretion and plasma values of 17 free and bound amino acids are being studied in conjunction with a clinical study to be reported elsewhere(2). The present paper summarizes the urinary excretion of free threonine, lysine, tyrosine,

and arginine in 41 patients before and after treatment.

**Experimental.** Thirty-one patients (12 ♂ and 19 ♀) were treated with ACTH and 10 patients (5 ♂ and 5 ♀) with Cortisone. All patients suffered from active rheumatoid arthritis and had been under observation for at least 6 months. They were hospitalized and controlled metabolically during the entire period of investigation. ACTH, given intramuscularly, in variable daily dosage (20 to 160 mg) was continued for a period of 8 to 17 days. Cortisone was given intramuscularly usually in an initial dose of 300 mg followed by 100 mg on successive days for about 10 days. All patients were not represented in determinations of free arginine and tyrosine. Various patients, receiving ACTH, were pretreated and treated simultaneously with one or more of the following in an effort to enhance or prolong the beneficial effect of ACTH: 6 were treated with thyroid and ascorbic acid, 4 with ascorbic acid alone, 6 with ATP (adenosine triphos-

\* This study was supported in part by a grant from the United States Public Health Service and The Fair Foundation. Cortisone was provided by Merck and Co. ACTH was provided by Armour and Co.

1. Stephens, C. A. L., Jr., Wallraff, Evelyn B., Borden, Alice L., Brodie, Emily C., Holbrook, W. Paul, Hill, Donald F., Kent, Leo J., Kemmerer, Arthur R., *Proc. Soc. Exp. Biol. and Med.*, 1950, v74, 275.

2. Holbrook, W. Paul, Stephens, C. A. L., Jr., Hill, Donald F., unpublished data.

## AMINOACIDURIA IN RHEUMATOID ARTHRITIS

TABLE I. Average and Maximum 24-hour Excretion of Free Threonine, Lysine, Tyrosine, and Arginine of Patients Treated with ACTH and ACTH Plus Other Medication.

Amino acid	Treatment	No. of patients	Mean of control	Mean of treatment	
			(mg)	Avg, mg	Maximum, mg
Threonine	ACTH	14	14 ± 1.95†	41 ± 5.68*	60 ± 10.02*
	ACTH + med.‡	17	19 ± 3.09	46 ± 13.68*	69 ± 21.32*
Lysine	ACTH	14	22 ± 3.32	52 ± 9.25*	78 ± 14.33*
	ACTH + med.	17	21 ± 2.86	43 ± 7.40*	58 ± 9.85*
Tyrosine	ACTH	12	18 ± 2.89	30 ± 3.54*	39 ± 4.39*
	ACTH + med.	7	23 ± 4.43	36 ± 6.75*	50 ± 8.59*
Arginine	ACTH	8	5 ± 0.98	6 ± 1.34	9 ± 1.50
	ACTH + med.	15	9 ± 2.26	9 ± 1.37	12 ± 1.66

\* Highly significant.

$$\dagger S_x = \sqrt{\frac{SX^2 - (SX)^2/N}{N(N-1)}}$$

‡ Supplementary medication.

TABLE II. Average and Maximum 24-hour Excretion of Free Threonine, Lysine, Tyrosine, and Arginine of Patients Treated with Cortisone.

Amino acid	No. of patients	Mean of control, mg	Mean of treatment	
			Avg, mg	Maximum, mg
Threonine	10	17 ± 1.63†	23 ± 2.13	29 ± 2.64*
Lysine	10	18 ± 4.54	32 ± 8.94	41 ± 10.78
Tyrosine	5	13 ± 2.45	21 ± 2.31	26 ± 2.36*
Arginine	4	6 ± 2.14	7 ± 2.63	9 ± 3.32

\* Highly significant.

$$\dagger S_x = \sqrt{\frac{SX^2 - (SX)^2/N}{N(N-1)}}$$

phate), 1 with X-ray, 1 with thyroid alone, 1 with aspirin, 1 with testosterone, and 3 were given 50 g of Somagen, protein hydrolysate. Fourteen patients were not given supplementary medication.

**Collection of urine specimens.** On alternate days during the experimental period 24-hour urine specimens were collected under toluene and diluted to appropriate volume. Aliquots were stored at -15°C. In all cases at least 2 specimens were obtained before ACTH or Cortisone medication was begun. A modification of microbiological technic of Henderson and Snell(3) was used for the assay of free amino acids. The test organism *Leuconostoc mesenteroides P-60* was used for estimating lysine and tryosine, and *Streptococcus faecalis-R* for arginine and threonine. All specimens from each patient were assayed in one

series to eliminate errors due to variation among different assays. An *in vitro* experiment was set up to determine whether medications administered might stimulate or inhibit the growth of the organisms. To 2000 ml of urine the following substances were added in the indicated amounts: ACTH, 1 to 160 mg; thyroid, 1 to 2 g; adrenalin, 1000 to 2000 units; testosterone propionate, 50 to 100 mg; ATP, 10 to 100 mg; ascorbic acid, 500 to 1500 mg. Microbiological assays were made on these specimens and no effect was noted.

**Results. Clinical.** All patients experienced both subjective and objective improvement within 24 to 48 hours following the initial injection of either ACTH or Cortisone and continued to improve during the course of treatment. The total degree of improvement varied among the patients. No toxic effects were noted except in the patient who received

3. Henderson, E. M., Snell, E. E., *J. Biol. Chem.*, 1948, v172, 15.

URINARY EXCRETION OF AMINO ACIDS BY PATIENT L.S.  
(♂ age 28, moderately severe rheumatoid arthritis) WHEN  
TREATED WITH ACTH AND CORTISONE RESPECTIVELY

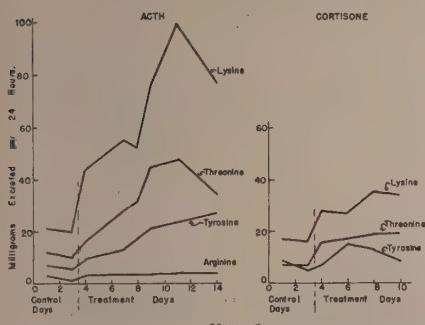


FIG. 1

160 mg ACTH daily. Each hormone produced the same type of response.

**Chemical.** Excretion values during the control periods were compared with average and maximum values on treatment. Data were analyzed statistically by Snedecor's analysis of variance(4). Patients receiving any medication in addition to ACTH were grouped together because so few patients were given each medication. This group was compared to that receiving ACTH only. No significant difference could be found between these two groups. Table I shows a highly significant increase in urinary excretion of free threonine, lysine, and tyrosine when patients were treated with ACTH, as calculated on the average and maximum 24-hour excretion. In Table II it is evident that the average 24-hour excretion of threonine, lysine, and tyrosine were not significantly increased while the maximum values of threonine and tyrosine are highly significant in

those patients treated with Cortisone. Maximum excretion of lysine was not significant. Arginine excretion was not affected significantly by either ACTH or Cortisone medication. No sex differences were observed. Supplementary medication did not significantly alter the patients' response to ACTH, either chemically or clinically.

The curves presented in Fig. 1 are typical of the responses to ACTH and Cortisone. These represent the daily excretion values of one man, L. S., 28 years of age, suffering from moderately severe rheumatoid arthritis, who received Cortisone in July and ACTH the following January. Usually the excretion curves, representing 8 or more days on treatment, show a decline from the maximum before the termination of treatment although the patient is still in remission.

**Summary and conclusions.** Rheumatoid arthritis patients treated with ACTH showed a highly significant increase in urinary free threonine, lysine, and tyrosine as determined as the average and maximum 24-hour excretion. Cortisone-treated patients excreted a highly significant amount of threonine and tyrosine at the maximum, but lysine was not increased significantly. Arginine excretion was not significantly affected by either ACTH or Cortisone. Supplementary medication did not affect significantly the responses of the patients to ACTH or Cortisone, chemically or clinically. Clinical improvement in all patients was both subjective and objective. The cause of the increase in urinary excretion of the amino acids herein reported is not known and may or may not be associated directly with the metabolic changes brought about by the remission of rheumatoid arthritis.

4. Snedecor, George W., *Statistical Methods*, Iowa State College Press, Ames, Iowa, 1948, 282.

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## Adequacy of Synthetic Diets for Reproduction of Swine. (18173)

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Previous efforts(1-4) to prepare synthetic diets that were adequate for reproduction by swine failed. Another attempt was made by the authors after vit. B<sub>12</sub> became available and the degree of success attained, though with only two sows, seems worthy of record. The experimental procedure has been described by Anderson and Hogan(4). The diet was of the synthetic type, homogenized with water to form a synthetic milk until the pigs were 10 weeks old. After that time the diet was fed as a dry mixture. The amount of casein was changed at times in order to better adapt the food mixture to the various stages of growth and reproductive activity, but in order to conserve space only the diets supplied during lactation are described in detail, see Table I. The experimental animals were two of the females described by Anderson and Hogan(5) and each bore two litters.

*First litters.* Each of the gilts had received injections of crystalline vit. B<sub>12</sub> at 3-day intervals beginning when they were 3 and ending when they were 38 days of age. The total amount injected in that period was 50 µg for No. 113 and 200 µg for No. 171. The gilts were bred at the ages of 195 and 238 days and farrowed when 312 and 354 days old. A solution of iron, copper and manganese was applied daily during the first 3 weeks of lactation to the udders of the sows to prevent anemia in their litters. When the

TABLE I. Composition of Experimental Rations.

Ration No.	358	359
Ingredient	%	%
Casein, vit. free*	25	22.5
Sucrose	55	55
Lard	5	5
Mineral mixture(6)	5	5
Wood pulp	10	10
Liver extract†	—	2.5
Vitamin supplement per 100 g of ration‡		
Thiamine-HCl	1	mg
Riboflavin	1	
Ca-pantothenate	3	
Pyridoxine-HCl	1	
Nicotinic acid	4	
Choline chloride	100	
Biotin	0.03	
Pteroylglutamic acid	0.02	
Inositol	100	
Vit. E	4	
Vit. K	2	
Vit. A	2000 I.U.	
Vit. D	400	

\* Labco, purchased from the Borden Company, New York, N. Y.

† Liver fraction primary powder purchased from Wilson Laboratories Division of Wilson and Company, Chicago, Ill.

‡ The pteroylglutamic acid was generously supplied by Dr. T. H. Jukes, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y. All other vitamins except A and D were generously supplied by Dr. D. F. Green of Merck and Co. The source of vit. A was a concentrate supplied by Distillation Products Co. of Rochester, N. Y. Vit. D was Delesterol purchased from E. I. du Pont de Nemours and Co.

pigs were 3 weeks old they had continuous access to the same feed in a creep as was supplied to their dams. When the sows were 231 days old the vitamin injections were resumed and continued at intervals until well into the lactation period. The total amounts injected during this time were 442 µg for Sow 113 and 383 µg for Sow 171. There was some slight evidence that the ration of the sows was inadequate. A few of the pigs were born with kinked tails, though this abnormality gradually disappeared. Mild diarrhea was observed in both litters but this condition was never severe or prolonged. Sow 171 was observed to vomit on a few occasions just prior to feeding. The farrowing and

\* Contribution from the Missouri Agricultural Experiment Station.

1. Ensminger, M. E., Bowland, J. P., and Cunha, T. J., *J. Animal Sci.*, 1947, v6, 409.

2. Russell, W. C., Terri, A. E., and Unna, K., *J. Nutrition*, 1948, v35, 321.

3. Hogan, A. G., and Anderson, G. C., *J. Nutrition*, 1938, v36, 437.

4. Anderson, G. C., and Hogan, A. G., *J. Animal Sci.*, 1950, v9, 163.

5. Anderson, G. C., and Hogan, A. G., *J. Nutrition*, 1950, v40, 243.

6. Richardson, L. R., and Hogan, A. G., *J. Nutrition*, 1946, v32, 459.

TABLE II. Farrowing and Litter Records.

Litter	First	Second	
Sow No.	113	171	113 171
Pigs born alive	7	8	8 9*
Avg birth wt, lb.	2.3	2.5	2.3 2.7
Creep feed consumed by litter, lbs.	n.w.†	n.w.†	132 129
No. of pigs weaned	6	6	7 7
Avg weaning wt, lbs.	20.4	25.8	35.1 47.1

\* A 10th pig was smothered in the chorionic membranes.

† n.w. = not weighed.

litter records of the two sows are summarized in Table II.

Two pigs in the litter of Sow 171 were dead by the 10th day and, as it seemed probable that the sow had received insufficient vit. B<sub>12</sub>, on the 11th day each pig was given an intramuscular injection of 21.5 µg of the vitamin. There were no more deaths. Each pig in the litter of Sow 113 was given an injection of 10 µg of vit. B<sub>12</sub> immediately after birth. There was one death in her litter at the age of 19 days which was attributed to an intestinal obstruction. It soon became evident that the pigs would not attain normal weaning weights, and an attempt was made to accelerate the growth rate. Some of the pigs in each litter were given additional injections of vit. B<sub>12</sub>, from 51.5 to 61.5 µg per pig, the others were given injections of the antipernicious anemia liver fraction,<sup>†</sup> from 80 to 95 units per pig. It was hoped that if pigs require an unrecognized nutrient it would be present in the liver extract and the existence of such nutrient could be established. The individual responses of the pigs, not shown, indicate that both vit. B<sub>12</sub> and the liver fraction improved the nutritional status of the pigs, but there was no evidence that the liver extract was any more effective than was the crystalline vitamin.

*Second litters.* The results of the first trial indicated that the sows had either been given an insufficient quantity of vit. B<sub>12</sub>, or else they require one or more unrecognized nutrients. In order to obtain additional information on these points both sows were car-

ried through a second reproductive period. In order to determine whether sows require more vit. B<sub>12</sub> than had been supplied in the first trial, Sow 171 was given 1.54 mg of vit. B<sub>12</sub> during gestation and 0.84 mg during lactation, a total of 2.38. Each pig in her second litter was given an injection of 50 µg of vit. B<sub>12</sub> shortly after birth. Sow 113 was used in the attempt to determine whether swine require an unrecognized nutrient. On the 59th day of gestation her ration was changed to Ration 359 which contains 2.5% of a water extract of liver.<sup>†</sup> When her litter was 6 weeks old the amount of the extract was increased to 5%. The pigs in the litter of Sow 171 were normal at all times. They were entirely free of the exudate on the body and around the eyes which previously has been so conspicuous(5,7) and they did not develop diarrhea. The litter was uniform, and the average weight at 8 weeks was 47.1 lbs., which is about 50% heavier than current growth standards for that age. It should be mentioned though that the pigs ate much more of the creep feed than they do when supplied with a mixture of the commonly used practical feed-stuffs, and this consumption of supplementary feed is probably the explanation of the unusual weaning weights. The performance of this litter would indicate that their diet contained all the vitamins that pigs require. The litter of Sow 113 did well, but its record was not spectacular. The average weight at 8 weeks was 35 lbs. However, the pigs had recurrent mild attacks of diarrhea during the first 3 weeks and they were not uniform in size. It is uncertain whether the differences between the two litters were due to their mothers or to the diets the mothers consumed. It may be that Sow 113 did not receive an adequate amount of vit. B<sub>12</sub>. Consideration should also be given to the fact that the litter of Sow 113 did not receive vit. B<sub>12</sub> injections. However the data are interpreted, they give no indication that the liver extract contains an unrecognized nutrient that is of importance to swine.

Our study was too limited for a precise

<sup>†</sup> We are indebted to Dr. R. A. Brown of Parke, Davis and Co., Detroit, Michigan, for this material.

7. McRoberts, V. F., and Hogan, A. G., *J. Nutrition*, 1944, v28, 165.

determination, but we have used the data obtained with Sow 171 for a tentative estimate of the amount of vit. B<sub>12</sub> required by a mature brood sow. The method of calculation is the same as was used by Anderson and Hogan(5) and the original data and the estimates are shown below.

Days in period	168
Feed consumed	558 kg
Casein consumed	103
Vit. B <sub>12</sub> in cascin	1,755 µg
Vit. B <sub>12</sub> inj.	2,380
Amt of vit. B <sub>12</sub> required per kg of feed	11.7
Amt of vit. B <sub>12</sub> required daily per sow:	
Orally	38.8
By injection	19.4

The estimate of the amount required per kg of feed is in reasonable agreement with an earlier estimate(5) of the amount required by a growing pig. One would expect both estimates to be revised when additional data become available.

*Discussion.* Rats, mice and chicks grow normally on diets that are completely synthetic. Guinea pigs and rabbits grow normally on synthetic diets but so far as we are aware normal reproduction has not been reported. Lambs and calves grow well, at least for limited periods, but have not been studied extensively. It seems probable that swine can be added to the list of animals for which synthetic diets are completely adequate. Admittedly it is desirable to have more animals but at present the cost is pro-

hibitive for us. However, it seems that two successes more than counterbalance several failures with less complete diets. Even if it is conceded though that synthetic diets are adequate, several nutritional factors remain for further investigation. The acceleration of growth by liver residues and by antibiotics, when added to certain rations, are well known examples.

*Summary.* Two sows which consumed synthetic diets and were given injections of approximately 400 µg each of vit. B<sub>12</sub> reared their first litters. The weights of the pigs when weaned at 8 weeks were subnormal, 20 and 26 lbs., and there were other signs of mild nutritional deficiency. One of the sows received by injection 2,380 µg of the vitamin during a second gestation and lactation and reared a litter of 7 pigs with the unusual average weaning weight of 47 lbs. The other sow consumed during her second gestation and lactation a diet that contained a water extract of liver and weaned a litter of 7 with an average weight of 35 lbs. The inclusion of a liver extract in the synthetic diet was not more effective than was the injection of vit. B<sub>12</sub> and the evidence indicates that swine can complete a normal life cycle on diets that contain no unrecognized nutrients.

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### Influenza Virus in Sectioned Tissues. (18174)

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As part of a broader study of how viruses develop within infected cells attempts are being made to recognize in thinly sectioned preparations the elementary particles of several viruses that have been examined in purified suspension. Photographs taken with this end in view have already been published of tobacco mosaic virus particles in infected leaves(1) and of particles of the fowl pox

virus(2) in infected chorioallantoic membrane. The present paper is a preliminary record of what has been seen in influenza-diseased tissues. It illustrates particles having the

1. Black, L. M., Morgan, C. and Wyckoff, Ralph W. G., Proc. Soc. EXP. BIOL. AND MED., 1950, v73, 119.
2. Morgan, C., and Wyckoff, Ralph W. G., J. Immunology, 1950, v65, 285.

dimensions of elementary bodies of influenza as seen in the chorioallantoic membranes of developing chicken embryos and in the lungs of adult mice infected with several strains of virus.

Most of the membranes have been from embryos used in the routine passage of virus strains. They were harvested after 48-72 hours incubation of inoculated eleven-day embryos. In a few experiments the membrane was taken after shorter periods of incubation. Membranes were immediately washed in saline and fixed in 4% neutral formalin-saline. They were then rinsed and pieces 2-3 millimeters on a side were dehydrated in alcohols or pyridine, embedded in methacrylate and cut as thin sections by the methods outlined by Neumann, Borysko and Swerdlow(3). Lung tissue was taken from mice used for virus passage. To obtain it four-weeks-old mice weighing 13 to 15 g each were inoculated nasally with the virus. Three days later they were sacrificed, the lungs were removed, fixed and small pieces from consolidated regions were processed in the fashion outlined above. After cutting, the thin sections of membrane or lung were mounted, methacrylate was removed, and they were then prepared for electron microscopy by the procedures followed in the examination of other virus-diseased tissues.

Allantoic fluid was tested from each infected embryonated egg supplying a chorioallantoic membrane for investigation. Without exception it agglutinated chicken erythrocytes in high dilutions. Virus infected mouse lungs similar to those examined under the electron microscope, when ground with alumnum, diluted and administered nasally to other mice, proved lethal in high dilutions. The lung lesions thus produced were typical of influenza virus infections. It has been harder to study the action of influenza virus on these tissues than it was to investigate fowl pox lesions because the immediate response of the susceptible cells to influenza is necrotic rather than hyperplastic. The severity of this necrotic reaction, however,

depends markedly on the strain. Using some of the less promptly destructive strains we have been successful in recording stages in cell disintegration and virus development. With these strains there is a progressive vacuolation of the cells of the membrane, an increase



FIG. 1

An electron micrograph of a section through a piece of chorioallantoic membrane infected with a recently isolated strain of A-type influenza. The mass at the bottom is cytoplasm of a border cell along the edge of which can be seen spherical and filamentous particles having the known diameter of influenza virus particles. The loss of detail within this cytoplasm is a mark of the developing necrosis. Magnification = 8,000 x.



FIG. 2

Filamentous and spherical particles of influenza in a suspension purified from allantoic fluid withdrawn from embryonated eggs diseased with the Weiss strain of A-type virus. Magnification = 11,333 x.

3. Neumann, S. B., Borysko, E., and Swerdlow, M., *Science*, 1949, v110, 66.

## INFLUENZA VIRUS IN SECTIONED TISSUES

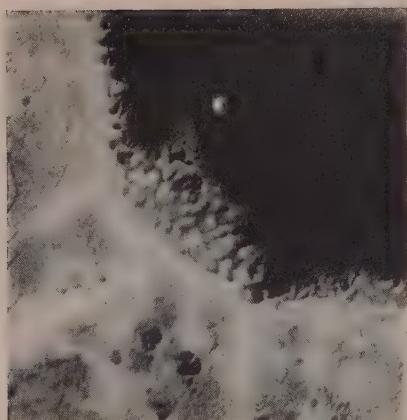


FIG. 3

Parts of the cytoplasm of three membrane cells with a large mass of virus-sized particles on the border of the middle cell and fewer particles along the right hand cell. Same virus strain as for Fig. 1. Magnification = 8,000 x.



FIG. 4

Isolated virus-sized particles in an alveolar space of a mouse lung infected with PR-8 virus. Particles are embedded in, and seemingly developing from, the remains of the cellular lining of the alveolar wall at the right. The somewhat variable diameters of the virus particles of these photographs are due in part to the varying amounts of adhering menstruum. Magnification = 8,000 x.

in the number of connective tissue elements in its mesodermal layer and sometimes even a minimal proliferation of the epithelial layer. Particles presumably of influenza are most readily recognized on the periphery of these cells before their vacuolation has progressed too far. One must imagine that with mem-

branes from the later stages of the infective process the virus already formed has been liberated into the allantoic fluid. A characteristic epithelial cell border with developing particles the size of influenza virus runs diagonally across Fig. 1. The similarity between these filaments and spheres and those found in purified suspensions is apparent from a comparison of Fig. 1 and 2. Clusters of shorter particles bordering a cell appear in Fig. 3. The particles can be seen deep within cells and even in dense clusters but usually they have seemed to be developing out of the cytoplasm of disintegrating cellular edges.

In mouse lung similar particles have been seen at the edges of consolidated areas. There the virus-like particles have been most often found disseminated through alveolar spaces, as in Fig. 4. The cells of the alveolar walls are so thin that it is difficult to see more than a small amount of a single cell in these preparations, but it would seem that virus develops from them as host cells; the cluster of particles associated with the wall at the right of Fig. 4 thus probably has grown from the cell that lined or constituted this wall.

These preliminary observations demonstrate that one can find particles in influenza diseased tissues that correspond in size and shape with those in purified suspensions of the virus. Similar particles have not been seen in healthy tissue and it accordingly seems legitimate to identify them with developing virus. If this is done it is clear that the influenza particles are growing at the expense of the cytoplasm of the infected cells. These photographs support previous evidence(4-9) that the filaments and spheres in the purified preparations are different aspects of virus

4. Mosley, V. M., and Wyckoff, Ralph W. G., *Nature*, 1946, v157, 263.
5. Heinmets, F., *J. Bact.*, 1948, v55, 823.
6. Dawson, I. M., and Elford, W. J., *Nature*, 1949, v163, 63.
7. Chu, C. M., Dawson, I. M., and Elford, W. J., *Lancet*, 1949, v1, No. 15, 602.
8. Isaac, A., Edney, M., Donnelley, M., and Ingram, M. W., *Lancet*, 1950, v1, No. 2, 64.
9. Murphy, J. S., Karzon, D. T., and Bang, F. B., *Proc. Soc. Exp. Biol. and Med.*, 1950, v73, 596.

and that some at least of the spheres may arise by segmentation of the long forms. Studies are being continued to learn more about the proliferation of this virus and to make more accurate observations on the obvious differences in the way strains of influenza attack and destroy the tissues they infect.

**Summary.** Particles having the known

dimensions of the influenza virus are observed in electron micrographs of thin sections cut from infected chorioallantoic membranes and mouse lungs. These particles are in groups and clusters apparently developing from the borders of membrane cells and from the walls of the alveoli.

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### Orotic Acid and Related Compounds in the Nutrition of *Lactobacillus bulgaricus* 09. (18175)

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Certain strains of *Lactobacillus bulgaricus* grow readily on a synthetic medium containing yeast extract as the source of an unknown nutritive essential (LBF)(1). Wright *et al.* reported(2) that one strain of *Lactobacillus bulgaricus*, identified as *Lactobacillus bulgaricus* 09, is incapable of growth on a basal medium supplemented with a tryptic digest of casein and yeast extract as crude components and requires much larger amounts of natural material such as milk products, liver or yeast to furnish another growth factor(s).

During the course of studies directed toward elucidating the nutritive requirements of *Lactobacillus bulgaricus* 09 it was found that orotic acid will replace the requirement for large amounts of natural material. When it became possible to grow *Lactobacillus bulgaricus* 09 with orotic acid in the basal medium described, studies were undertaken to determine the extent to which the norit-treated tryptic digest of casein and the yeast extract contributed to the growth factor requirements of the organism. It has been found that the yeast extract is a dispensable component of the medium provided adequate

amounts of the norit-treated tryptic digest of casein are present. The growth factor contributed by the norit-treated tryptic digest of casein has been identified with "streptogenin"(3). With the availability of a basal medium that promotes good growth of *Lactobacillus bulgaricus* 09 when supplemented with orotic acid, data have been obtained with respect to (a) the extent to which certain compounds can substitute for orotic acid and (b) the distribution of orotic acid. As a consequence of these findings it has been possible to make certain deductions concerning possible pathways of pyrimidine synthesis in nature and the possible role of orotic acid in nucleic acid metabolism.

**Experimental.** *Lactobacillus bulgaricus* 09 was obtained from Dr. I. C. Gunsalus to whom we are indebted. The organism was maintained in stock culture by weekly transfer in Difco skim milk to which 1% Difco tryptose was added. For the preparation of inocula day to day transfer of the organism in the milk-tryptose medium with return to stock culture at weekly intervals was carried out. For seeding microbiological assays 0.1 ml of a 24-hour culture in milk-tryptose medium was dispersed with shaking into 10

1. Williams, W. L., Hoff-Jorgensen, E., and Snell, E. E., *J. Biol. Chem.*, 1949, v177, 933.

2. Wright, L. D., Huff, J. W., Skeggs, H. R., Valentik, K. A., and Bosshardt, D. K., *J. Am. Chem. Soc.*, 1950, v72, 2312.

3. Wright, L. D., Fruton, J. S., Valentik, K. A., and Skeggs, H. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1950, v74, 687.

## OROTIC ACID AND PYRIMIDINE SYNTHESIS

TABLE I. Basal Medium.\*

Norit-treated acid-hydrolyzed casein	1.0	g
Norit-treated tryptic digest of casein	0.5	
Sodium acetate	1.2	
Glucose	4.0	
L-Tryptophane	20	mg
L-Cystine	20	
Adenine	1	
Guanine	1	
Xanthine	1	
Uracil	1	
Salts A	1	ml
Salts B	1	
Thiamine chloride	200	γ
Riboflavin	200	
Calcium pantothenate	200	
Nicotinic acid	200	
Pyridoxine	400	
Pyridoxal	50	
PABA	100	
Folic acid	50	
Biotin	1	
Vit. B <sub>12</sub>	0.04	
Tween 80	0.2	ml
pH to 5.7 and dilute to	100	

\* Concentrations given are for double strength medium.

ml of sterile saline. One drop per tube of this saline suspension was the inoculum used. 37°C was the incubation temperature employed. The basal medium used in these studies had the composition given in Table I.

Turbidimetric measurement of bacterial density with the Klett-Summerson photoelectric colorimeter or titration of the lactic acid produced against 0.1 N NaOH with bromthymol blue as indicator were used as alternative methods for measuring the extent of bacterial growth. Seventy-two hours was the usual incubation time employed. Other microbiological procedures were in accordance with standard methods(4). The orotic acid used in these studies was synthetic material(5,6) (Fig. 2). The orotic acid and intermediates studied had ultraviolet absorption spectra identical with those published by Mitchell and Nyc(5,6). The miscellaneous compounds tested for possible orotic acid activity were, except as noted, commercial or personal research samples.

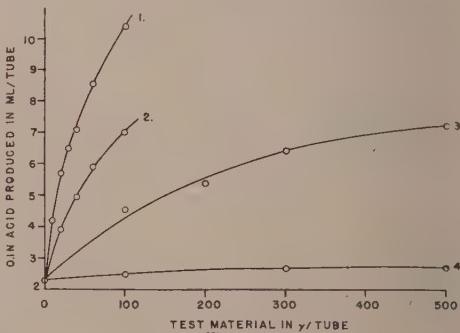
4. Snell, E. E., Vitamin Methods, 1950, v1, Academic Press, Inc., New York, New York.

5. Mitchell, H. K., and Nyc, J. F., *J. Am. Chem. Soc.*, 1947, v69, 674.

6. Nyc, J. F., and Mitchell, H. K., *J. Am. Chem. Soc.*, 1947, v69, 1382.

**Results. Microbiological activity of orotic acid and related compounds.** The response of *Lactobacillus bulgaricus* 09 to orotic acid is shown in Fig. 1. The same response is obtained in the absence of uracil from the basal medium. Also included in Fig. 1 are the data obtained on the microbiological activity of ureidosuccinic acid, 5-(acetic acid)-hydantoin, and 5-(carboxy-methylidine)-hydantoin. These compounds are obtained as intermediates in the synthesis of orotic acid by two accepted methods for the synthesis (Fig. 2) of this compound starting with diethyloxaloacetate and urea(5) or aspartic acid(6). 5-(Carboxy-methylidine)-hydantoin (V) has 30-40% of the activity of orotic acid, ureidosuccinic acid (II) is 10-20% as active, while 5-acetic acid hydantoin (III) is essentially inactive. To obviate the possibility that 5-(carboxy-methylidine)-hydantoin was converted partially to orotic acid during autoclaving, the compound also was tested following sterile filtration and aseptic addition to previously autoclaved media. The same results were obtained by the two methods of assay. 5-(Acetic acid)-hydantoin at levels up to 5000 γ/tube does not inhibit utilization of either 5-(carboxy-methylidine)-hydantoin or ureidosuccinic acid.

Compounds\* that have been found to be inactive at levels up to at least 100 γ/tube include: adenosine, adenosine-3-phosphoric acid, adenosine-5-phosphoric acid,<sup>1</sup> β-alanine, allantoin, alloxan, γ-amino-butyric acid,<sup>2</sup> 2-



The response of *Lactobacillus bulgaricus* 09 to orotic acid and related compounds, (1) orotic acid (VI), (2) 5-(carboxy-methylidine)-hydantoin (V), (3) ureidosuccinic acid (II), and (4) 5-(acetic acid)-hydantoin (III).

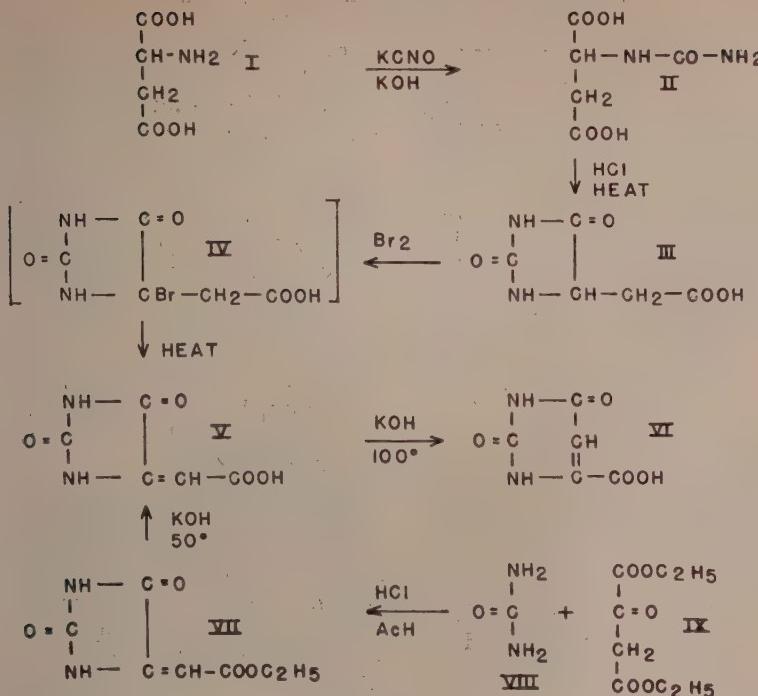


FIG. 2

Structures of orotic acid and intermediates involved in the synthesis of orotic acid by 2 methods (5,6). I aspartic acid, II ureidosuccinic acid, III 5-(acetic acid)-hydantoin, IV 5-bromo-5-(acetic acid)-hydantoin, V 5-(carboxy-methylidine)-hydantoin, VI orotic acid, VII 5-(carboxy-methylidine)-hydantoin, VIII urea, IX diethyl oxaloacetate.

amino-4-methyl-6-oxyurimidine,<sup>3</sup> asparagine, aspartic acid, benzimidazole, caffeine, 5-carboxy-uracil,<sup>4</sup> choline, citrulline, creatine, creatinine, cytidine, cytidylic acid, cytosine, 2, 4-diamino-6-oxyurimidine, desoxyribonucleic acid, fumaric acid, fumaric acid plus urea, guanidine, guanosine, guanylic acid, hypoxanthine, indole-3-acetic acid, inositol,

lactose, maleic acid, maleic acid plus urea, malic acid, malic acid plus urea, N-methyl-3-carboxylamide-2-pyridone, N-methyl-3-carboxylamide-6-pyridone, N-methyl-3-carboxy-6-pyridone, 1, 7-dimethyl-5-oxy-(1,5-dihydro-1,6-naphthyridine), 2-methyl-5-ethoxymethyl-6-aminopyrimidine, 4-methyl-uracil,<sup>5</sup> 5-methylthiouracil, nicotinamide, ornithine, oxaloacetic acid, o-phenylene urea,<sup>5</sup> pteric acid,<sup>6</sup> 4-pyridoxic acid, 2-pyrolidone-5-carboxylic acid, pyruvic acid, ribonucleic acid, succinic acid, theobromine, theophylline, thymidine,<sup>7</sup> thymine, trigonelline, uracil, urea, uric acid, uridine,<sup>8</sup> uridylic acid, xanthopterin,<sup>3</sup> xanthurenic acid.<sup>3</sup>

Thymidine, uridylic acid, and cytidylic acid alone or in combination have no activity when tested in the presence of an amount of orotic acid permitting partial growth of *Lactobacillus bulgaricus* 09.

A concentrate of *Lactobacillus bulgaricus*

\* We are indebted to the following individuals for the indicated miscellaneous compounds:

<sup>1</sup> Dr. H. A. Lardy, University of Wisconsin.

<sup>2</sup> Dr. Karl Folkers, Merck & Company, Inc.

<sup>3</sup> Dr. J. M. Sprague and coworkers, Sharp & Dohme, Inc.

4. Dr. H. K. Mitchell, California Institute of Technology.

5 Dr. J. O. Lampen, formerly of American Cyanamid Co.

<sup>6</sup> Dr. E. L. R. Stokstad, Lederle Laboratories, Inc.

<sup>7</sup> Dr. E. E. Snell, University of Wisconsin.

<sup>8</sup> Dr. H. S. Loring, Stanford University.

TABLE II. Apparent Orotic Acid Content of Natural Materials.

Sample	Orotic acid, γ/g or ml
Delactosed whey	2600
Skim milk powder	580
Whey nutrient I	600
," ", II	600
Corn steep solids	345
Yeast extract	2670
Liver fraction "S"	1600
," ", "L"	2000
Dried distillers' solubles	1040
Dried mold mycelium	280
Urine I	94
," II	110
," III	84
," IV	76

factor (LBF)(1) from Dr. E. E. Snell was found to be inactive in lieu of orotic acid for *Lactobacillus bulgaricus* 09.

*The Occurrence of Orotic Acid in Natural Materials.* A variety of natural materials have been assayed for orotic acid following solution or dispersion in water. The orotic acid content of various natural materials, subject to the limitations mentioned in the Discussion, is summarized in Table II.

*Discussion.* The ability of ureidosuccinic acid and 5-(carboxy-methylidine)-hydantoin to replace orotic acid in the nutrition of *Lactobacillus bulgaricus* 09 is unexpected. The existence of an equilibrium between 5-(carboxy-methylidine)-hydantoin and orotic acid such that 5-(carboxy-methylidine)-hydantoin would appear to have 30-40% of the activity of orotic acid is quite unlikely under the conditions of microbiological assay employed. Acid conditions would appear to influence any such equilibrium in favor of the hydantoin rather than the pyrimidine since an hydantoin instead of a pyrimidine is obtained when ureidosuccinic acid is treated with strong acid. The microbiological activity of ureidosuccinic acid is difficult to explain on the basis of any equilibrium phenomenon. It is suggested that 5-(carboxy-methylidine)-hydantoin is utilized after hydrolysis to ureidofumaric acid. The apparent microbiological activity of ureidosuccinic acid and 5-(carboxy-methylidine)-hydantoin may indicate that the synthesis of the pyrimidine ring in nature involves cyclization of an aliphatic ureido dicarboxylic acid

such as ureidosuccinic acid or ureidofumaric acid.

It should be appreciated that any data on the distribution of a component of natural material is subject to the specificity of the method of assay employed. It would appear, however, from the data of Table II that orotic acid or a microbiologically active related compound has an ubiquitous occurrence in nature. In the case of milk products the orotic acid content has been confirmed by actual isolation of the compound in essentially quantitative yield (see accompanying paper)(7). Other natural materials with high orotic acid activity by microbiological assay are, at present, under study.

The fate of orotic acid in the nutrition of *Lactobacillus bulgaricus* 09 is at present unknown but it may be assumed that it is involved in nucleic acid synthesis. Since uridine, cytidine, uridylic acid and cytidylic acid as well as uracil and cytosine are inactive in lieu of orotic acid with *Lactobacillus bulgaricus* 09, it is conceivable that the synthesis of nucleic acid, at least in this organism, does not involve the participation of uracil or the uracil nucleosides and nucleotides but that orotic acid becomes incorporated into nucleic acid before decarboxylation. Such an hypothesis is not incompatible with recent data, obtained from isotopic studies, indicating that orotic acid but not uracil or uridine is a precursor of nucleic acid pyrimidine in the rat(8-11).

*Summary.* The orotic acid requirement of *Lactobacillus bulgaricus* 09 can be met by ureidosuccinic acid (10-20% as active as orotic acid) or by 5-(carboxy-methylidine)-hydantoin (30-40% as active as orotic acid) but not by any one of a large group of re-

7. Huff, J. W., Bosshardt, D. K., Wright, L. D., Spicer, D. S., Valentik, K. A., and Skeggs, H. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1950, v75, 297.

8. Arvidson, H., Eliasson, N. A., Hammarsten, E., Reichard, P., and Ubisch, H. V., *J. Biol. Chem.*, 1949, v179, 169.

9. Hammarsten, E., Reichard, P., and Saluste, E., *J. Biol. Chem.*, 1950, v183, 105.

10. Reichard, P., *Acta chem. Scand.*, 1949, v3, 422.

11. Hammarsten, E., Reichard, P., and Saluste, E., *Acta chem. Scand.*, 1949, v3, 433.

lated or unrelated compounds studied. The data are taken as microbiological evidence that ureidosuccinic acid and possibly ureido-fumaric acid (derived from 5-(carboxy-methylidine)-hydantoin) are precursors of the

pyrimidine ring.

Data on the distribution of orotic acid in nature are presented.

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### A Growth-Promoting Substance for *L. bulgaricus* 09 in Whey: Isolation and Identification as Orotic Acid. (18176)

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In 1949 Williams *et al.*(1) reported the presence of a factor in yeast extract required by certain strains of *Lactobacillus bulgaricus* for growth in a synthetic medium. During investigations in our laboratories on the growth requirements of several strains of the same species, it was found that one strain identified as *Lactobacillus bulgaricus* 09 was incapable of growth when the medium was supplemented with yeast extract but required a factor present in liver, milk products or other natural materials(2). It was found in a survey of known compounds that orotic acid (4-carboxy uracil) was capable of replacing the requirement of the organism for the natural material(2). Of the natural materials tested, whey was found to be a very good source of the growth-promoting factor. This source was selected for isolation studies to investigate the possible identity of the microbiological activity of whey with orotic acid. The isolation of the microbiological activity from this natural source and its characterization as orotic acid are presented in this communication.

**Experimental.** Assays for the factor were carried out microbiologically using *Lactobacillus bulgaricus* 09 as described in the accompanying paper(3). Orotic acid was

used as a standard in the assay and the activities of the various fractions obtained are expressed in terms of this standard. 1.25 kg of powdered whey\* were stirred for one hour with 25 liters of water and the mixture filtered with the aid of 1.2 kg of Hyflo filter aid. 21.3 liters of a yellow aqueous extract were obtained. This solution contained by microbiological assay 92 γ/ml of the factor expressed as orotic acid or a total of 1.94 g of activity. The aqueous extract was stirred for ½ hour at pH 3 with 426 g of Norit A; filtered using suction on a 12" Buchner funnel and washed with approximately 3 liters of water. 0.18 g out of 1.94 g of activity remained in the charcoal filtrate. An elution of the substance was affected by allowing 1 N sodium hydroxide solution to pass slowly through the charcoal bed employing suction. Twelve 300-ml fractions were collected. A total of 1.7 g of activity were eluted as indicated by microbiological assay of the fractions. Fractions No. 5 through 10 which contained 1.01 g of activity were pooled, neutralized to pH 7 with concentrated hydrochloric acid and concentrated under vacuum to 300 ml. The dark yellow concentrate which contained at this point about 23% sodium chloride was allowed to stand overnight in the refrigerator. After filtering and washing with cold water and with alcohol there were obtained 1.2 g of grayish white crystalline material subsequently identified

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\* "Edible Quality" spray dried delactosed whey was purchased from the National Dairy Laboratories, Oakdale, Long Island, N. Y.

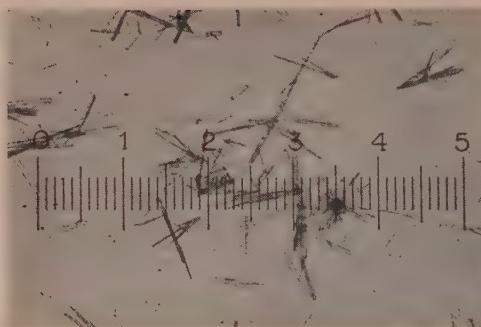


FIG. 1a

(a) Crystals of the sodium salt of orotic acid. (b) Crystals of orotic acid. Scale; 0 to 1 = 113 microns.



FIG. 1b

as a sodium salt. This material contained about 90% orotic acid as indicated by microbiological assay. About 9.6 mg of activity remained behind in the filtrate. In the presence of high concentrations of sodium chloride the sodium salt of orotic acid will crystallize readily as rather well defined needles even from solutions containing large amounts of impurities. The sodium salt also is obtained from strong acid solutions (pH 1) which contain high concentrations of salt.

The 1.2 g of the crude crystalline sodium salt were boiled for  $\frac{1}{2}$  hour with 125 ml of water and filtered while hot to remove 160 mg of an extremely water-insoluble microbiologically inactive crystalline compound. About 200 mg of Darco G-60 were added to the slightly yellow filtrate and after stirring for a few minutes the hot mixture was filtered and the colorless solution placed in the refrigerator overnight. After filtration, the crystalline mass was washed with water and alcohol and dried over  $P_2O_5$ . 670 mg of snow-white crystalline material were obtained. The crystals were well defined long needles as shown in Fig. 1a. An additional 230 mg of the crystalline salt were obtained by concentration and cooling of the filtrate. Thus of the 1.01 g of orotic acid activity indicated by microbiological assay to be present in the pooled charcoal eluates selected for purification, 900 mg actually were obtained in crystalline form. This material does not contain water of crystallization but is, however, slightly hygroscopic. 300 mg of the crystal-

line sodium salt were dissolved in 35 ml of boiling water and 0.4 ml of concentrated HCl added. On cooling overnight in the refrigerator 220 mg of the free acid were obtained as white crystalline plates as shown in Fig. 1b. The crystalline acid contains 1 molecule of very tightly bound water of crystallization.

A comparison of the sodium salt and the free acid of the isolated material was made with synthetic orotic acid<sup>†</sup> and its sodium salt and the results are summarized in Table I.

A micropotentiometric titration of 10 mg of the isolated acid with 1.39 N sodium hydroxide confirmed a molecular weight of 174 and indicated the presence of 2 acidic groups having pK values of 2.46 and 9.40 respectively as shown in Fig. 2. Similar data were obtained for synthetic orotic acid.

Ultraviolet absorption curves on the sodium salts dissolved in water at a concentration of 20  $\gamma/ml$  were made on the Beckman quartz spectrophotometer and showed an absorption maximum at 278  $m\mu$  and a minimum of 240  $m\mu$ . The corresponding molecular extinction coefficients are shown in Table I.

The isolated material and synthetic orotic acid were equivalent in their ability to promote growth of *Lactobacillus bulgaricus* 09 when assayed under the conditions described in the accompanying paper(3).

*Discussion.* The data presented in Table I indicate that the growth promoting factor for *Lactobacillus bulgaricus* 09 present in

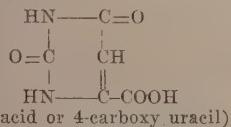
<sup>†</sup> Orotic acid was prepared from oxalacetic ester and urea according to the method described by Mitchell and NYC(4).

TABLE I. Comparison of Properties of Orotic Acid and Growth-promoting Factor for *Lactobacillus bulgaricus* Isolated from Whey.

Compound	M.W. by titration	pK <sub>1</sub>	pK <sub>2</sub>	$\epsilon_{278 \text{ m}\mu}$	$\epsilon_{240 \text{ m}\mu}$	$\epsilon_{278}/\epsilon_{240}$	% N*
Isolated compound	177	2.46	9.40	7130	1730	4.11	15.73
Orotic acid	180	2.51	9.40	7180	1710	4.19	—
Theoretical	174	—	—	—	—	—	15.73

\* This analysis was carried out on the sodium salt of the isolated compound.

whey is identical with orotic acid. This compound has the structure shown in the accompanying diagram.



It is believed that practically all of the growth-promoting activity of the water extract of whey is due to the presence of orotic acid in view of the almost quantitative recovery of the activity as the crystalline compound. In the survey of other compounds ureidosuccinic acid and 5-(carboxy-methylidine)-hydantoin were found to replace orotic acid in the nutrition of *Lactobacillus bulgaricus* 09. These two substances are discussed in the accompanying paper(3).

Orotic acid was discovered and isolated from cow's milk in 1905 by Biscaro and Belloni(6). This substance was found by

Loring and Pierce to replace cytosine and uracil in satisfying the growth requirements of 2 pyrimidine-deficient mutants of *Neurospora*(7). Mitchell *et al.* have reported that orotic acid is produced in large amounts by certain mutant strains of *Neurospora* and suggest that possibly this substance arises from a side reaction in the biosynthesis of nucleic acid(5). Chattaway reported that orotic acid can replace the folic acid requirement of certain microorganisms, indicating a possible role of this compound in the biosynthesis of nucleic acid(8). Recent isotopic studies with rats suggest that orotic acid rather than uracil is a precursor of nucleic acid pyrimidine(9-12).

**Summary.** Various natural substances especially whey were found to contain a substance required for growth of *Lactobacillus bulgaricus* 09. In the survey of known compounds orotic acid was found to satisfy the requirement of this organism for natural material. The microbiologically active material was isolated from one of the natural sources, whey, and identified with orotic acid.

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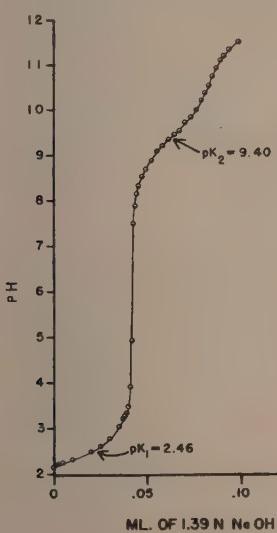


FIG. 2.

Titration curves of 10 mg (0.0574 mm) of crystalline orotic acid isolated from whey.

# Influence of Concentration of the Vitamin B Complex on Protein Efficiency of Blood Fibrin.\* (18177)

BARNETT SURE.

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In a recent communication(1) it was demonstrated that with casein as the protein in the ration, increasing the concentration of various components of the vitamin B complex resulted in marked increases in growth and pronounced increases in food utilization. However, increases in nitrogen retention and protein synthesis occurred only within a narrow range of the low concentrations of the B vitamins. The marked increases in growth were due largely to fat synthesis from the carbohydrate (cerelose) in the rations. On a low 7.1% casein level some growth was possible but only when the various components of the vitamin B complex were raised to high concentrations. On the same low protein intake and on low concentrations of the B vitamins, only maintenance was possible. In the study referred to the Wistar strain albino rats were fed *ad libitum* and the increased growth was associated with increased food intake, stimulated by the higher concentrations of the vitamin B complex components.

In this investigation blood fibrin (Armour) having a protein content of 91% was chosen

TABLE I. Composition of Vitamin B Complex Mixtures.

	1	2	3
Thiamine	5 γ	15 γ	25 γ
Riboflavin	5	15	25
Pyridoxine	5	15	25
Niacin	5	15	25
Calcium pantothenate	50	100	150
p-Aminobenzoic acid	500	2 mg	3 mg
Inositol	150	600 γ	1
Choline chloride	6 mg	6 mg	6

for study. Three concentrations of the B vitamins were used and the food intake was controlled to the maximum that was consumed daily by each animal, which was 6 g.

The rats were started on experiments when about 28 days of age and each weighed about 50 g. The rations consisted of various levels of blood fibrin; cellu flour, 2; Sure's salts No. 1(2), 4; vegetable shortening, 8; cod liver oil, 2; wheat germ oil, 1; and the rest of the rations was the carbohydrate, cerelose. The composition of the vitamin B complex (VBC) mixtures are given in Table I and referred to as VBC 1, 2, and 3. The results are summarized in Table II. The blood

TABLE II. Influence of Concentration of Vitamin B Complex on Protein Efficiency of Blood Fibrin Controlled Food Intake. (Avg results of 8 animals on each group, 4 ♂'s and 4 ♀'s).

Protein	VBC mixtures	Protein in ration, %	Total food intake, g	Experimental period, days	Gain in body wt, g	Protein intake, g	Protein efficiency ratio*	
							Increase, %	
Blood fibrin 9.1% protein	1	10	456	76	41.0	41.5	.99 ± .14†	
	2	10	456	76	49.7	41.5	1.20 ± .13	21.2
	3	10	456	76	57.3	41.5	1.38 ± .11	41.4
13.65% protein	1	15	456	76	52.5	62.3	.84 ± .21	
	2	15	456	76	76.3	62.3	1.22 ± .07	45.1
	3	15	456	76	76.9	62.3	1.23 ± .17	46.2
18.2% protein	1	20	456	76	43.5	83.0	.52 ± .17	
	2	20	456	76	69.7	83.0	.84 ± .10	61.5
	3	20	456	76	66.1	83.0	.80 ± .07	53.9
	1	20	582	97	45.0	105.9	.42 ± .16	
	2	20	582	97	76.1	105.9	.72 ± .08	71.4
	3	20	582	97	77.7	105.9	.73 ± .05	73.8

\* Gain in body wt per g protein intake.

† Stand. dev. of the means.

intake, introduced 9.1, 13.65, and 18.2% fibrin, fed at 10, 15, and 20% planes of protein in the rations, respectively.

It will be noted from Table II that, on the 10% blood fibrin ration, during an experimental period of 76 days, on the same 456 g food intake, a change from VBC 1 to VBC 2 produced an increase of 21.2% in the protein efficiency ratio (PER), as indicated by gains in body weight per gram of protein intake; and a change of VBC 1 to VBC 3

produced an increase of 41.4% in the PER. On the rations containing 15 and 20% blood fibrin, on the same food intake, there were pronounced increases in the PER, i.e., an increase of 45.1 to 71.4%, when the VBC 1 was changed to VBC 2, but no further increases in PER when VBC 1 was further increased to VBC 3.

**Summary.** When blood fibrin (Armour) was the protein in the ration, on controlled food intake, increasing the concentration of mixtures of various components of the vitamin B complex, resulted in marked increases in the protein efficiency ratio, expressed as gains in body weight per gram of protein intake.

\* Research paper No. ....Journal Series, University of Arkansas. Published with the approval of the Director of the Arkansas Agricultural Experiment Station.

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## Beta-Hyperglobulinemia Produced by Cholesterol Feeding in the Rabbit.\* (18178)

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Recent work(1) has focused attention on the possibility that deposition of cholesterol in atherosclerosis is correlated, not with analytically demonstrable hypercholesterolemia, but rather with the circulation of relatively large aggregates of cholesterol or cholesterol combined with protein as a lipoprotein. That clinical hypercholesterolemia is often accompanied by hyperglobulinemia, including rise in beta-globulin, has been found in diabetes, obstructive jaundice and myxedema. Dubach and Hill(2) have shown analytically that the hypercholesterolemia produced in rabbits by cholesterol feeding is accompanied by increase in serum globulin. The experiments herein reported show that the hyperglobulinemia resulting from chole-

sterol feeding in rabbits is due almost entirely to increase in beta-globulin.

**Procedure.** 28 rabbits 6 to 8 weeks old were fed a basic diet of Rockland Farms pellets for periods varying between 5 weeks and 27 months. The cholesterol ration for each week was dissolved in ether and poured over the pellets. Following evaporation of the ether, the rabbits readily ate the cholesterol-coated pellets. The rabbits consumed between 3 and 20 g of cholesterol per week. Total protein, albumin and globulin were determined by precipitating with 23%  $\text{Na}_2\text{SO}_4$  followed by micro-Kjeldahl as in(3). Electrophoretic separations were made at pH 8.56 in barbiturate buffer. Cholesterol was determined by a modification of the method of Bloor, Pelkan and Allen(4). Readings were made at wave length 420 mu. Phospholipid

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## INDUCED BETA-HYPERGLOBULINEMIA

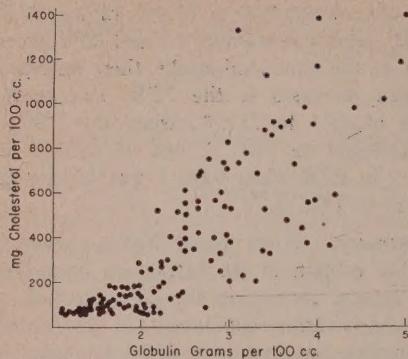


FIG. 1a

Relation of serum cholesterol level to concentration of albumin (b) and globulin (a) in 28 rabbits.

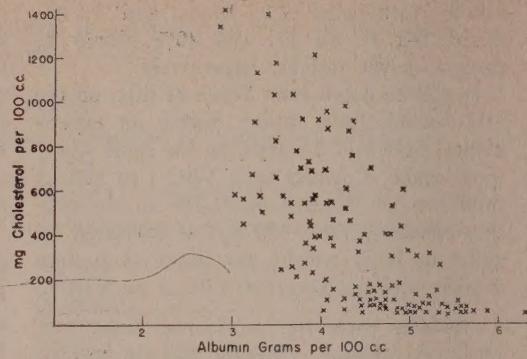


FIG. 1b

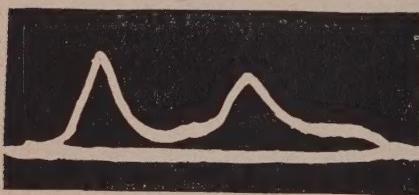
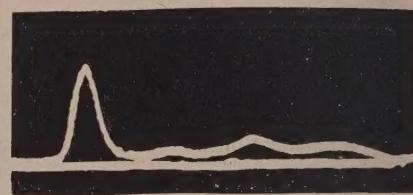


FIG. 2  
Electrophoretic patterns of sera of Rabbit 573.  
1/9/50

Cholesterol 1413 mg %  
Serum proteins (rel. %)

Albumin +	$\alpha_1$ -globulin	39.8
"	$\alpha_2$ -	5.7
"	$\beta$ -	40.3
"	$\gamma$ -	14.2



2/28/50  
Cholesterol 256 mg %  
Serum proteins (rel. %)

47.7
10.8
22.3
19.2

was determined by the method of Youngberg and Youngberg(5).

**Results.** In Fig. 1a the cholesterol content of the serum is plotted against the globulin and in Fig. 1b against the albumin level. As cholesterol rose with continued feeding, globulin increased and albumin diminished.

The electrophoretic pattern of Rabbit 573 was typical (Fig. 2). It shows that the rise in serum globulin accompanying hypercholesterolemia is due almost entirely to beta-globulin. Jan. 9, when cholesterol feeding had elevated serum cholesterol to 1413 mg/100 ml, beta-globulin constituted 40.3% of total protein; Feb. 28, after discontinuation of cholesterol feeding had been followed by fall in serum cholesterol to 216 mg/100 ml, beta-

globulin was 22.3% of total protein.

The rise in serum cholesterol in rabbits produced by cholesterol feeding is accompanied by elevation in phospholipid (Table I). However, the proportionately greater rise in cholesterol, as has been previously observed(6), results in an augmented cholesterol/phospholipid ratio.

In 2 dogs fed 10 to 20 g of cholesterol 6 days weekly for 9 and 11 months, respectively, there were relatively slight changes in serum cholesterol, phospholipid, albumin and globulin. However, each of these slight changes was in the same direction as in the rabbit.

**Discussion.** The observations throw no light on the mechanism by which rise in serum cholesterol entails rise in beta-globulin and fall in albumin. The findings harmonize with previous suggestions that beta-globulin

TABLE I. Changes in Lipid and Protein Fractions During and After Cholesterol Feeding (Rabbit #42. Lipids mg, proteins g/100 cc.).

	3/3*	4/14†	5/15
Total cholesterol	91	1312	450
Free ,,,	30	352	209
Ester ,,,	61	960	241
Phospholipids	50	325	155
Total fatty acids	922	2383	1164
Total protein	6.05	5.76	5.55
Albumin	3.47	2.33	3.05
Alpha <sub>1</sub> -globulin	.49	.39	.20
Alpha <sub>2</sub> -globulin	.56	.52	.26
Beta-globulin	.86	1.99	1.40
Gamma-globulin	.60	.51	.59
Cholesterol/phospholipids	1.82	4.03	2.90
Albumin/globulin	1.3	.66	.91

\* Cholesterol feeding started.

† Cholesterol feeding discontinued.

may function as a carrier for lipids in the form of a lipoglobulin. Autopsy on the rab-

bits fed cholesterol disclosed the anticipated severe atherosclerosis. In view of the large size of the beta-globulin molecule, the rise in beta-globulin which accompanies the hypercholesterolemia in these animals, with resultant cholesterol-beta-globulin complexes, accords well with those theories(1,7) which attribute atherosclerosis to the circulation and deposition in the arterial wall of giant cholesterol-containing aggregates.

**Summary.** The hyperglobulinemia accompanying hypercholesterolemia produced by cholesterol feeding in the rabbit is predominantly due to beta-globulin.

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### Effect of *p*-Aminobenzoic Acid and Vitamin C Upon Duration of Survival of Nephrectomized Rats. (18179)

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In studying the rate of acetylation of *p*-aminobenzoic acid (PABA) in nephrectomized rabbits (to be published) it was observed that these animals uniformly died 3 to 4 days following removal of the second kidney. Each had received an intraperitoneal injection of 40 to 65 ml of 5% glucose solution containing 200 to 1000 mg of PABA, neutralized to pH 7.4 with NaOH, 18 to 24 hours after the second nephrectomy (ether anesthesia). After withdrawal of a series of small blood specimens from an ear vein they were returned to cages where food (Purina rabbit chow) and water were available *ad lib*. Because methemoglobin formation occurred in an occasional animal, Vit. C in amounts from 50 to 500 mg was added to the fluid injected into another group of similarly treated rabbits, and it was at once noticed that the duration of survival was strikingly increased, ranging from 5½ to 8½ days. Even more strik-

ing was the improved condition of the animals during most of the period of survival. They were alert, active, and in most respects behaved like normal rabbits until a few hours prior to death. These apparent effects of a single dose of a combination of PABA and Vitamin C have since been confirmed by an independent observer upon a limited number of nephrectomized rabbits (Dr. E. E. Muirhead; personal communication).

Inasmuch as careful records had not been kept in connection with these casual observations it was decided to study a series of rats under more controlled conditions to determine whether any increase in survival time after nephrectomy is caused by the administration of vitamin C, alone, or whether the combination of PABA and the vitamin is required.

**Experimental.** Forty-eight male albino rats weighing from 314 to 417 g, and maintained

on a diet of Rockland rat ration, were unilaterally nephrectomized. About 3 weeks later the second kidney was removed from the entire series (ether anesthesia). The animals were then divided into 4 groups which received intraperitoneal injections immediately after operation as follows: (a) *Control Group* (12 rats). These were given a volume of salt solution ranging from 5.0 to 5.8 ml containing that amount of sodium in the neutralized Vitamin C-PABA mixture given to group (d); i.e. 4.28 meq NaCl/kg. (b) *Vit. C Group* (12 rats). These were given 100 mg/kg Vitamin C, in volumes of neutralized solution ranging from 1.6 to 1.8 ml. (c) *PABA Group* (12 rats). These were given 200 mg/kg PABA, in volumes of neutralized solution ranging from 3.2 to 4.2 ml. (d) *PABA plus Vit. C Group* (12 rats). These were given Vitamin C, 100 mg/kg, and PABA, 200 mg/kg in volumes of neutralized solution ranging from 1.5 to 1.8 ml. The animals were returned to their cages where food and water were available *ad lib.*, and checked for survival at frequent intervals subsequently so that the time of death could be estimated to the nearest hour. No particular differences were observed in food and water consumption of the 4 groups.

**Results.** The results are summarized in Table I. The mean survival time of Group (d) receiving the combination of Vitamin C and PABA was 21.5% greater than that of the control Group (a), the differences being statistically significant. The increase of mean survival time of Group (c), which received only PABA was border-line in significance. Vit. C, alone, Group (b), had no effect upon survival.

**Discussion.** It is our impression that the survival and general condition of the nephrectomized rat are less affected by administration of a combination of Vit. C and PABA than are those functions in the rabbit. In this series of rats it appears that PABA alone may slightly increase survival whereas no such effect of PABA alone has been observed

TABLE I. Hours of Survival After Nephrectomy by Rats Treated with Vitamin C and PABA.  
Survival time, hr ( $\pm$  0.5 hr).

Rat No.	Group (a) control	Group (b) vit. C	Group (c) PABA	Group (d) vit. C + PABA
1	47.5	46	47	56
2	49.5	48	51	60
3	49.5	48	51	61
4	49.5	50	55	61
5	49.5	50	59	64
6	53.5	54	65	64
7	55.5	54	65	72
8	55.5	58	65	72
9	55.5	60	75	76
10	59.5	60	77	76
11	76.5	71	82	80
12	80.5	79	87	86
Mean (hr)	56.8	56.5	64.9	69.0
S.D.	10.3	9.6	12.7	8.9
Diff. of means*		-0.3	8.1	12.2
2X S.D. of diff. of means*		8.12	9.34	7.86

\* Compared to Group I as control.

in rabbits. The difference might possibly be due to more active intestinal synthesis of Vit. C by the rat, in which case the group receiving PABA alone was also obtaining some Vit. C as well. Vit. C alone was clearly without effect.

It is recognized that many variables may contribute to survival time after nephrectomy; e.g. environmental temperature, water intake, total caloric intake, mineral intake (Na,K), etc. The casual observations referred to above upon rabbits were so striking in the absence of obvious operation of such variables that the increase in survival was interpreted as a specific effect of the combination of Vit. C and PABA. It seems likely also that the less pronounced effect in rats is, nevertheless, a specific one. Further inquiry is being made into these findings.

**Summary.** The administration of a single dose of a combination of Vit. C and *p*-aminobenzoic acid to nephrectomized rats significantly increases the survival time.